REMARKS

Claims 97-99, 101-111 and 113-118 are pending in the application. Applicants have herein above amended independent claims 97, 111, and 113. Claims 98 and 99 are canceled without prejudice in light of the amendments to claims 97, 111 and 113. The claim amendments are completely supported by the application as originally filed, and thus they do not involve any issue of new matter. Therefore, entry of this amendment is respectfully requested such that claims 97, 101-111 and 113-118, as amended, will be pending.

Applicants appreciate the courtesies extended by the Examiner during a telephone conference with their representative, John P. White, Esq. (Reg. No. 28,678), on March 26, 2002. The remarks set forth herein are in accordance with the matters discussed during the subject telephone conference.

Based on the telephone conference, it is applicants' understanding that this amendment is to be filed in the Office by telefacsimile, and that the Examiner will give this response an expedited review. Following such review it is applicants' further understanding that the Examiner will permit an interview with applicants' counsel to discuss, inter alia, the above amendments in an effort to resolve any remaining issues concerning the patentability of the claims of the present case, which issues may also be relevant to several pending related applications by the same inventors. If this understanding is not correct, the Examiner is respectfully requested to telephone applicants' representative at the number below to clarify any such misunderstanding.

Applicants note with appreciation the statement in ¶5 on p.2 of the Office Action that the rejection of claims 97-99 and 101-118 under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention, is withdrawn in light of the amendments previously made to the claims.

Objection to the Disclosure

The Examiner stated that the prior objection to the disclosure is maintained for the reasons set forth in the Office Action mailed June 18, 1998 (Paper No. 16). The Examiner further stated that applicants submit they will provide a new Figure 6B to overcome the rejection when the case is in condition for allowance. The Examiner additionally stated that until applicants submit a proper figure, the objection is maintained.

In response, applicants will provide a new Figure 6B upon the indication of allowable subject matter.

Obviousness Type Double Patenting Rejection

The Examiner provisionally rejected claims 97-99, 101-111 and 113-118 as being unpatentable due to obvious-type double patenting over claims 78-92 and 94-99 of copending Application No. 08/477,097 for the reasons made of record in Paper No. 20 mailed October 6, 1999, and Paper No. 22, mailed June 27, 2000. The Examiner stated that applicants' arguments filed August 1, 2001, i.e., that the claims of Application No. 08/477,097 do not render obvious the instant claims, have been fully considered but are not persuasive because applicants

have provided no reasoning to dispute the obviousness set forth in the previous Office Actions.

The Examiner has additionally provisionally rejected claims 97-99, 101-111 and 113-118 as being unpatentable due to obviousness-type double patenting over pending claims 78-93 and 95-100 of copending Application No. 08/475,084 [Sic. 08/475,784] for the reasons made of record in Paper No. 20, mailed October 6, 1999 and Paper No. 22, mailed June 27, 2000. The Examiner stated that applicants' arguments filed August 1, 2001, i.e., that the claims of 08/475,784 do not render obvious the instant claims, have been considered but are not persuasive because applicants have provided no reasoning to dispute the obviousness set forth in previous Office Actions.

In a new ground of rejection, as set forth in ¶11 on p.12 of the Office Action, claims 97-99, 101-111 and 113-118 are provisionally rejected as being unpatentable due to obviousness-type double patenting over claims 109 122 of copending Application No. 08/477,147. The Examiner stated that, although the claims are not identical, they are not patentably distinct from each other because the claims of Application No. 08/477,147 also encompass the same composition as that which is instantly claimed (i.e., a conjugate comprising a ganglioside derivative with an altered ceramide portion conjugated to an immunogenic protein based carrier, a saponin and a pharmaceutically acceptable carrier), and a method of treatment using such.

The provisional double-patenting rejections of claims 97-99, 101-111 and 113-118 of the present application over applications Serial No. 08/477,097; 08/475,784 and 08/477,147 are respectfully traversed. In response to these rejections,

applicants submit that M.P.E.P. §804 IB, in discussing provisional double-patenting rejections between copending applications, requires that the:

> 'provisional' double patenting rejection should continue to be made by the examiner in each application as long as there are conflicting claims in more than one application unless that 'provisional' double patenting rejection is the only rejection remaining in one of the applications. If the 'provisional' double patenting rejection in one application is the only rejection remaining in the application, the examiner should then withdraw that rejection and permit the application to issue as a patent, thereby converting the 'provisional' double patenting rejection in the other application into a double patenting rejection at the time one application issues as a patent. (emphasis supplied by applicants).

Applicants submit, therefore, for the reasons discussed below, that the claim amendments made herein to claims 97, 111 and 113 are believed to overcome the §103(a) rejection of those claims, as well as the claims which depend therefrom, which rejections should therefore be withdrawn. Following such withdrawal of the §103(a) rejections, the only remaining rejection in this application would be the provisional double patenting rejection of claims 97-99, 101-111 and 113-118. In accordance with the M.P.E.P. section quoted above, the provisional double patenting rejection should thus be withdrawn to permit the application to issue as a patent. Such action is therefore respectfully solicited.

Rejection Under 35 U.S.C. 103 (a)

The Examiner stated that the prior rejection of claims 97-99, 101-111 and 115-118 under 35 U.S.C. §103(a) as being unpatentable over Livingston et al (Cancer Research, 149:7045-7050,1989) in view of Ritter, et al. (Seminars in Cancer Biology, 2:401-409, 1991), Liane et al. (Journal of Biological Chemistry, 249 (14):4460-4466, 1974), Livingston et al. (U.S. Patent No. 5,102,663), Ritter et al. (Immunobiol, 812:32-43, 1990), Kensil et al. (The Journal of Immunology, 146(2):431-437, 1991), Marciani et al. (Vaccine, 9:89-96, 1991) and Uemura et al. (J. Biochem, 79(6):1253-1261, 1976) is maintained for the reasons made of record in the "previous Office Actions". The Examiner then reiterated these reasons as follows.

Examiner's summary of bases for claim rejections:

The Examiner stated that Livingston et al (Cancer Research) teach a composition administered to melanoma patients for stimulating the production of antibodies directed against a carbohydrate epitope on the ganglioside GM2 (page 7046 7048). The Examiner stated that Livingston et al. teach that the composition for treatment is administered at concentrations of 100, 200 or 300 µg with an adjuvant, Bacillus-Calmetre-Geurin (BCG), and a pharmaceutically acceptable vehicle, phosphate buffered saline, p. 7046, column 1, paragraph 3, and paragraph bridging p. 7046-7047.). The Examiner stated that Livingston et al. teach that melanoma recurrence was delayed in patients developing GM2 antibodies after treatment with the composition (page 7048, paragraph 1 and column 2, paragraph 2). The Examiner stated that Livingston et al. teach that more patients produce IgM antibodies than IgG antibodies to the GM2 (page 7047, paragraph bridging columns 1-2). The Examiner also

stated that Livingston et al. also teach the gangliosides GM2, GD2 and GD3 are expressed on the cell surface of human malignant melanomas (page 7045, column 1, paragraph 2) and that Livingston et al. differ [i.e., from the present invention] by not teaching the conjugation of the GM2 or other gangliosides by means of a carbon on the ceramide moiety with aminolysyl groups on Keyhole Limpet Hemocyanin (KLH) in a composition and using this composition for treatment.

The Examiner further stated that Ritter et al (1991) teach that IgG response to gangliosides may be increased by the covalent attachment of foreign carrier proteins such as KLH to the ganglioside, resulting the in the T cell help necessary for the response (page 406, paragraph 1). The Examiner stated that Ritter et al. teach that the advantage of including an IgG antibody response (vs IgM) against gangliosides is that IgG: a) has a higher affinity, b) is better able to penetrate solid tissues, c) is able to mediate antibody-dependent cell-mediated cytotoxicity, and d) is generally detectable in the serum for longer periods after immunization.

The Examiner additionally stated that Liang et al. (Journal of Biological Chemistry, 249(14):4460-4466, 1974) teach a method for covalent coupling of gangliosides to aminoethyl agarose or the amino group-bearing glass beads by oxidative ozonolysis of the olefinic bond of the sphingosine moiety (i.e., the instant carbon double bond of ceramide) and coupling of the carboxyl bearing product to the amino group of aminoethyl agarose or the amino group-bearing glass beads.

The Examiner also stated that Ritter et al. (1990) teach that GD3 lactone is more immunogenic than GD3.

The Examiner additionally stated that Livingston et al. (U.S. Patent No. 5,102,663) teach that gangliosides GM3, GM2, GD3, GD2, GT3 and O-acetyl GD3 are gangliosides that are prominent cell-membrane components of melanoma and other tumors of neuroectodermal origin (column 1, lines 22-28).

The Examiner further stated that Kensil et al. teach that QS-21 (i.e., the instant carbohydrate derivable from the bark of a Quillaja saponaria Molina tree) produced a higher antibody response than conventional aluminum hydroxide (page 433, column 2, paragraph 4, and Figure 3). Kensil et al. also teach that the immune responses obtained with QS-21, reached a plateau at doses between 10-80 µg in mice (page 433, column 1, paragraph 3).

The Examiner additionally stated that Marciani et al. teach the use of Q3-21 adjuvant was useful because it did not cause toxic reaction in cats (page 93, paragraph 1).

The Examiner additionally stated that Uemura et al. (J Biochem, 79(6):1253-1261, 1976) teach that the ozonolysis and reduction of various sphingolipids did not affect the haptenic reactivity of the ganglioside derivative with antibodies.

The Examiner therefore stated that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to modify the composition taught by Livingston et al. by conjugating the GM2 to KLH by covalently coupling GM2 to KLH by substituting GM2 for the globoside and KLH for aminoethyl agarose to produce a GM-2-KLH conjugate by means of the olefinic bond of the sphingosine moiety of the GM2 (i.e., the instant ceramide double bond) and the ε-aminolysyl groups present in the KLH protein using the method

of Liang et al. (emphasis supplied by applicants) and to add QS-21 as an adjuvant to the GM2-KLH conjugate for use as a vaccine because the conjugated composition would be expected to enhance the IgG response to the ganglioside, as taught by Ritter et al. (1991), thus providing the advantages by Ritter et al. (1991) and adding the QS-21 would be advantageous because it provides for a higher antibody response that the commonly used adjuvant used by Kensil et al. and QS-21 provides the advantages that it is not toxic to animals as is taught by Marciani et al.

The Examiner therefore stated that it would also have been *prima facie* obvious to use doses of between 10 and 80 µg of QS-21 in the composition and to optimize the dose accordingly because the immune response with QS-21 plateaus at doses between 10-80 ug and optimization of the weight ratio of the components of the composition to provide an optimal response is well within the ordinary skill in the art as is use the composition as modified supra for treatment of melanoma as taught by Livingston et al. (Cancer Research).

The Examiner additionally concluded that it would also have been *prima facie* obvious to one of ordinary skill in the art to substitute any one of GM3, GD2, GD3, or 9-acetyl GD3 for the GM2 ganglioside in the composition and method as combined, supra, because they are all prominent cell-membrane components of melanomas as taught by Livingston et al. (U.S. Patent No. 5,102,663) and one of ordinary skill in the art would know they react with the melanoma cells.

The Examiner further stated that it would also have been prima facie obvious to one of ordinary skill at the time the invention was made to substitute the GD3 lactone for the GM2

ganglioside in the composition because GD3 lactone is more immunogenic than GD3, as taught by Ritter et al. (1990) and would be expected to product an enhanced antibody response as compared to GD3.

The Examiner further stated that optimization of the dosage, the route of immunization, and the number of sites of immunization to administer the composition, are well within the skill of the ordinary artisan.

The Examiner further stated that one would have reasonably expected the conjugation procedure to work as substituted because conjugation through the ε-aminolysyl groups of carrier proteins for enhanced immunogenicity is routine in the art and Uemura et al. (J Biochem, 79(6):1253-1261, 1976) teach that the ozonolysis and reduction of various sphingolipids did not affect the haptenic reactivity with antibodies.

Examiner's analysis of arguments made by applicants to claim rejections in their August 1, 2001 response:

The Examiner stated in the Office Action that applicants argue that the references do not teach, suggest or disclose applicants invention. The Examiner further stated that specifically, applicants' argue that the primary reference, Livingston et al. (1990) fails to teach conjugation of GM2 or other gangliosides by means of a carbon on the ceramide moiety with aminolysyl groups on KLH in a composition, or using this conjugate for treatment, and that applicants further argue that the secondary references fail to supply this teaching.

The Examiner stated that with regard to Ritter et al. (1991), applicants acknowledge that Ritter et al. (1991) teaches conjugation of GM2 to KHL. The Examiner further stated that

applicants argue that Ritter et al. (1991) fails to teach the chemical nature of the GM2-KLH conjugate, or how to make the conjugate, and further that the reference does not disclose conjugation through the ceramide.

The Examiner additionally stated that with regard to Ritter et al. (1990), applicants argue that there is no teaching of conjugation to KLH, and further, that modifications of the gangliosides of Ritter et al. (1990) are in the carbohydrate portion, not the ceramide portion, such that Ritter et al. (1990) teach away from ceramide conjugation.

The Examiner additionally stated that with regard to Liang et al., applicants supplied Helling et al., which applicants argue teaches that Liang et al. method "is of limited use for the conjugation of ganglioside to carrier proteins because it requires acetylated, methyl ester derivatives of gangliosides to avoid coupling via the sialic acid carboxyl group. Deacylation after conjugation under basic conditions is necessary, conditions most proteins cannot be exposed to without degradation". The Examiner stated that, based on this teaching, applicants concluded that Liang et al. fails to supply the missing teachings of the primary reference. The Examiner further stated that with regard to the other secondary references (Uemura et al., Konsil, et al., Marciani et al., and Livingston et al. (U.S. Patent 5,102,663)) applicants argue that these references fail to teach a ceramide linkage.

The Examiner went on to state that applicants' arguments filed August 1, 2001 in response to these grounds for rejection have been fully considered but they are not persuasive.

Examiner's response to applicants' arguments in their August 1, 2001 response:

Responding to applicants' arguments in their August 1, 2001 response (see, e.g., pp.10-16 of the subject Amendment, which remarks will not be repeated here), the Examiner stated in the present Office Action that the conjugate and method of treatment taught in Livingston et al. teaches the instantly claimed conjugate, but fails to teach conjugation to KLH.

The Examiner further stated in the Office Action that Ritter et al. (1991) teaches that the conjugation of GM2 to KLH is desirable because it generates a superior immune response, and that with regard to Ritter et al. (1991), applicants' argument that the reference fails to teach the specific ceramide conjugation is not persuasive because such a conjugation was known in the art at the time the invention was made (as set forth in the additional secondary references). The Examiner additionally stated that the key teaching of Ritter et al. (1991) is that one would expect a superior immune response when GM2 is coupled to KLH. The Examiner stated that Ritter et al. (1991) provides motivation to conjugate the ganglioside to KLH.

The Examiner stated that, with regard to Ritter et al. (1990), applicant's arguments misrepresent the teachings of Ritter et al.(1990) and the Examiner's reasons for citing such. According to the Examiner, Ritter et al. (1990) was cited for the teaching that GD3 lactone is more immunogenic that GD3 and that the reference was not cited to represent ceramide linkage.

The Examiner additionally stated that in contrast to the applicants' arguments, Liang et al. does not require

deacetylation after conjugation. The Examiner stated that it appears that the reaction that applicants had referred to is that of figure 2 in the Liane et al. paper, in which the deacetylation step occurs after glass beads have been conjugated to the ganglioside. The Examiner in her remarks then pointed applicants to figure 1 of Liane et al., which contains a different reaction, i.e., one which provides carbodiimide linkage under standard acidic, not basic conditions. The Examiner stated that the deacetylation step in the conjugation method of figure 1 occurs before the linkage step and the protein is not present in basic conditions when substituted for the sepharose. The Examiner further stated that carbodiimides under conditions of Liane et al. have long been used for the coupling of peptides to carrier proteins and will not degrade the protein, and that with regard to the other secondary references (Uemura et al., Kensil et al., Marciani et al. and Livingston et al. (U.S. Patent 5,102,663)) that applicants only argue that these references fail to teach a ceramide linkage. The Examiner stated, however, that they (i.e., the secondary references) are not cited for the teaching of a ceramide linkage. The Examiner thus stated that the rejection is maintained for reasons of record.

The eight reference cited in combination to reject claims 97-99, 101-111, 113 and 115-118 under 35 U.S.C. §103(a) are all extensively discussed by applicants in their submission filed August 1, 2001 (see, e.g., pp 12-16 of the August 1, 2001 submission). Those discussions, which applicants believe provide sufficient grounds for distinguishing the invention over the cited references, will not be repeated here. However, the substance of the subject arguments is expressly incorporated into this response by reference thereto.

Responding, therefore, to the grounds for rejection as summarized by the Examiner in the present Office Action, Applicants respectfully traverse the Examiner's rejection that the invention recited in the claims is obvious over the cited art. Applicants respectfully disagree with the Examiner's contention that the conjugation procedure described by the references as combined provides the same procedure as applicants' presently claimed coupling procedure. Applicants contend that the cited references, namely Livingston et al. (Cancer Research) in view of Ritter et al. (Seminars in Cancer Biology), Liang et al.(Journal of Biological Chemistry), Livingston et al. (U.S. Patent No. 5,102,663), Ritter et al. (Immunobiol), Kensil et al. (The Journal of Immunology), Marciani et al. (Vaccine) and Uemura et al. (J. Biochem) do not teach, suggest, or otherwise disclose applicants' claimed invention and therefore these references, in combination, do not render obvious the claimed invention.

In support of their position, Applicants submit that the presently pending independent claims of the application (nos. 97, 111, and 113) are now amended to recite, respectively a composition and methods involving administration of the composition, wherein the composition comprises: a) a conjugate of i) a GM2 ganglioside derivative which comprises an unaltered oligosaccharide part and an altered ceramide portion comprising a sphingosine base, to ii) Keyhole Limpet Hemocyanin, comprising an ε-aminolysyl group; b) a saponin derivable from the bark of a Quillaja saponaria Molina tree; and c) a pharmaceutically acceptable carrier; the relative amounts of such conjugate and such saponin being effective to stimulate or enhance antibody production in a subject, **wherein in the conjugate the ganglioside derivative is covalently bound to the Keyhole Limpet Hemocyanin through a C-4 carbon of**

the sphingosine base of the ceramide portion of the ganglioside derivative to the ε-aminolysyl group of Keyhole Limpet Hemocyanin, wherein the C-4 carbon is present in a CH_2 group. [Emphasis added].

As noted by the Examiner in the paragraph bridging pp.5-6 of the present Office Action, Liang et al,. (Journal of Biological Chemistry, 204 (14):4460-4466, 1974) teach a method for covalent coupling of gangliosides to aminoethyl agarose or amino group-bearing glass beads by oxidative ozonolysis of the olefinic bond of the sphingosine moiety (i.e., the instant carbon double bond of the ceramide) and coupling of the carboxyl bearing product to the amino group bearing glass beads.

On p. 10 of the Office Action, the Examiner specifically points applicants to Figure 1 of the Liang, et al. reference (see p.4461). As illustrated therein the ganglioside is coupled to the amino group through a C-4 carbon which forms part of a C=O group.

In contrast, and as now specifically recited in independent claims 97, 111 and 113 of the present application, the ganglioside derivative of the composition of the present invention is covalently bound, i.e. conjugated, to the Keyhole Limpet Hemocyanin, through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative to the ε-aminolysyl group of Keyhole Limpet Hemocyanin, wherein the C-4 carbon is present in a CH_2 group. [emphasis added].

The above-described linkage is clearly illustrated in Figure 1-2 of the present application wherein the C-4 carbon through

which the covalent bonding occurs forms part of a CH_2 group, and not a C-O group as in the case of the Liane, et al. reference. As the presently claimed mode of linkage is neither taught nor even suggested by the Liane et al. reference, nor any of the other references cited in combination to rejection claims 97, 101-111, 113 and 115-118, applicants respectfully submit that the invention as now recited in the (amended) independent claims, as well as the claims which depend therefrom, is not obvious to one of ordinary skill in the art. Thus, the rejection of claims 97, 101-111, 113 and 115-118 under 35 U.S.C. §103(a) should be withdrawn. Claims 98-99 have been canceled (without prejudice) as noted above.

Rejection Under 35 U.S.C. §103(a)

The prior rejection of claim 114 under 35 U.S.C. §103(a) as being unpatentable over Livingston et al. (Cancer Research), Ritter et al. (Cancer Biology, 1991), Liane et al. (Journal of Biological Chemistry, 249 (14);4460-4466 (1974), Livingston et al., (U.S. Patent No. 5,102,663), Ritter et al. (1990), Kensil et al. and Marciani et al. and Uemera et al. (J. Biochem., 79(6):1253-1261, 1976) as applied to claims 97-99, 101-111, 113 and 115-118, and further in view of Irie et al. (U.S. Patent No. 4,557,931) is maintained by the Examiner, for reasons of record "in previous Office Actions" and which were reiterated as follows.

The Examiner stated that the combination differs by not teaching the administration of the composition for treating cancer of epithelial origin.

With regard to the patent to Irie et al., the Examiner stated that Irie et al. teaches that the ganglioside GM2 is found on

or in tumors of a variety of histological types including melanoma and breast carcinomas (column 1, lines 28-31).

The Examiner concluded in the Office Action that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to administer the GM2-KHL conjugate/QS-21 composition or other ganglioside conjugate/QS-21 composition as combined *supra* to patients afflicted with or susceptible to a recurrence of cancer of an epithelial origin (i.e. breast carcinomas) because the ganglioside GM 2 is found in the stroma of the tumor as taught by Irie et al. and one of ordinary skill in the art would expect that the antibodies produced by the composition react with the tumor and treat the disease.

The Examiner noted that Applicants argue that Irie et al. does not supply the missing teaching of a ceramide linkage and that Applicants arguments filed August 1, 2001 have been fully considered but they are not persuasive.

The Examiner further stated that the teaching of a ceramide linkage is not missing, and Irie et al. is not relied upon to teach such. The Examiner additionally stated that Irie et al. teach that the ganglioside GM2 is found on or in tumors of a variety of histological types including melanoma and breast carcinomas (column 1, lines 28-31) and that Applicants have provided no arguments for such. The Examiner thus stated that the rejection is maintained for the reasons of record.

Applicants respectfully traverse the rejection of claim 114 in that claim 114 is dependent from claim 113 which, as discussed above, is clearly distinguishable over the combination of Livingston et al., Ritter, et al. (Seminars in Cancer Biology, 1991), Liane et al.), Livingston et al. (U.S. Patent No. 5,102,663), Ritter et al. (1990), Kensil et al., Marciani et al., and Uemura et al. in view, inter alia, of the mode of conjugation recited in claim 113, i.e., between the ganglioside derivative and the Keyhole Limpet Hemocyanin, which occurs by a covalent bond through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative to the ε aminolysyl group of the Keyhole Limpet Hemocyanin, wherein the C-4 carbon is present in a CH_2 group. [emphasis supplied]. None of the above-cited references teach or even suggest such a linkage to one of ordinary skill in this art.

Applicants contend, moreover, that Irie, et al. does not satisfy the element(s) missing from the above-discussed references, and thus does not remedy the deficiencies of those references. Irie et al. is simply cited, as noted in the Office Action, for its teaching that the ganglioside GM2 is found on or in tumors of a variety of histological types, including melanoma and breast carcinomas. There is no teaching or suggestion in Irie et al. as to the claimed mode of conjugation as presently recited in all of the independent claims, including claim 113 from which claim 114 depends as noted above.

Accordingly the references as applied to claims 97-99, 101-111 and 115-118 above and further in view of Irie et al. (U.S. Patent No. 4,557,931) do not teach, suggest or disclose

applicants' claimed invention and therefore the combination does not render obvious the claimed invention. Applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Additional support for the patentability of claims 97-99, 101-111, 113-118 is provided by the disclosure of several publications, which provide evidence of unexpected immunobiological results achieved with the use of compositions as recited in the subject claims. Further with regard to those claims, applicants note that the independent claims 97, 111 and 113 have each been amended in subparagraph (a) to recite only the GM2 ganglioside, and to delete the recitation of the GD2 ganglioside. These amendments are not for the purpose of overcoming the prior art, but rather they have been made to prevent any overlap with claims of one or more related applications to the present case.

Of particular interest is a review article by P. Livingston, Ganglioside Vaccines with Emphasis on GM2, Seminars in Oncology, Vol. 25, no.6 (December), 1998, pp. 636-645, attached hereto as **Exhibit B**. The article states, at p. 641 (in col. 1, ¶1), that:

> Keyhole limpet hemocyanin (KLH) was the best of the six immunogenic carrier molecules tested in the mouse, the method of conjugation was crucial, and a potent immunologic adjuvant was required.
> ...A variety of different carriers and adjuvants have also been tested with gangliosides GM2, GD2 and fucosyl $GM1^{2,36}$. In each case, the ganglioside covalently attached to KLH via the ceramide moiety

plus QS21 induced the highest titers of IgM and IgG antibodies.

The particular conjugation (between the ganglioside and the KLH) used with the present invention is described in detail in Helling et al., Cancer Research, Col 54:197-203 (1994) (using GD3 as the ganglioside derivative). A copy of the subject reference is attached hereto as **Exhibit C**. The relevant disclosure is found at p. 198, col.1, ¶5, i.e. "GD3 Conjugate Preparation", and in Fig. 1 on p. 199.

Additional disclosures relating to the claimed conjugation arrangement is found in Helling et al., Cancer Research, Vol. 55:2783-2788 (1995), attached as **Exhibit D**, wherein GM2 is used as the ganglioside derivative (see, e.g., p. 2783, col. 1, ¶2). According to the reference:

> Briefly, the conjugation procedure involved ozone cleavage of the ceramide double bond of GM2, introduction of an aldehyde group, and conjugation to aminolysyl groups of KLH by reductive animation.

As is seen from the teachings of the present application, of which both Messrs. Livingston and Helling are co-inventors, use of the conjugation procedure as outlined in the reference above results in conjugation of the ganglioside to the KLH through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative (e.g., GM2) to the ε-aminosyl group of the KLH, wherein the C-4 carbon is present in a CH_2 group (i.e., and not as a C=O group as disclosed in the Lianc et al. reference cited in combination to reject applicants' claims).

Further to the above, in clinical trials melanoma patients vaccinated with GM2 KLH and QS21, made using the conjugation procedure described and claimed in the present application, produced high titre IgM and IgG antibodies specific for GM2. Moreover, in at least one-half of the patients, the anti-GM2 antibody response persisted for more than 5 ½ months. Support for this is found, e.g., in Table 2 of Exhibit A (at p. 640), Table 2 of Exhibit C (at p.2787), as well as in Chapman, et al; Clinical Cancer Research, Vol 6:874-879 (March 2000), attached hereto as **Exhibit E**.

As these cited references provide clear and unambiguous evidence of unexpected improvements in immunological results achieved by the composition and methods of applicants' presently pending claims, and further as the scope of the subject claims is commensurate with the evidence provided thereby, applicants submit that this evidence clearly supports an allowance of the subject claims over the prior art cited by the Examiner.

Applicants additionally note the statement, at §12 on p.13 of the Office Action, that the prior art made of record, i.e., Harlow and Lane, Antibodies-A Laboratory Manual, Chapter 6, pp. 84-85 (1988), while not relied upon is considered pertinent to applicants' disclosure. In response thereto, applicants submit that they have reviewed the cited reference and that it neither teaches nor suggests the invention as presently recited in the claims, whether taken, alone or in combination with any other(s) of the cited references.

Supplemental Information Disclosure Statement

In compliance with their duty of disclosure under 37 C.F.R. §1.56, applicants direct the Examiner's attention to the following reference, which is listed on accompanying form PTO-1449 (**Exhibit F**), a copy of which is attached hereto as **Exhibit G.**

Price, V.L., U.S. Patent No. 5,616,477, issued April 1, 1997, filed July 7, 1994. (Exhibit G).

This reference was cited in an Office Action dated September 7, 1999 in a related application (Serial No. 08/481,809) to the present application. Applicants maintain that the subject reference neither discloses nor suggests the invention claimed in the present application, whether viewed alone or in combination with any of the other cited references.

A fee of ONE HUNDRED EIGHTY DOLLARS ($180.00) is believed due for submission of this Information Disclosure Statement. Authorization is hereby provided to charge the required fee to Deposit account No. 03 3125.

Summary

For all of the reasons set forth hereinabove, applicants respectfully request that the Examiner reconsider and withdraw the various grounds of objection and rejection and earnestly solicit allowances of the now pending claims, i.e., claims 97, 101-111 and 113-118.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' attorney

invites the Examiner to telephone at the number provided below.

A $460.00 fee for a three-month extension of time, together with a $180.00 fee for submission of the Information Disclosure Statement, for a total of SIX HUNDRED FORTY DOLLARS ($640.00) is deemed necessary in connection with the filing of this response. Authorization is hereby given to charge the amount of the required fee to Deposit Account No. 03-3125.

Respectfully submitted,

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Exhibit A

Exhibit A

Amended Claims

--97. (twice amended) A composition which comprises:

a) a conjugate of i) a GM2 [or GD2] ganglioside derivative which comprises an unaltered oligosaccharide part and an altered ceramide portion comprising a sphigosine base, to ii) Keyhole Limpet Hemocyanin, comprising an ε-aminolysyl group;

b) a saponin derivable from the bark of a Quillaja saponaria Molina tree; and

c) a pharmaceutically acceptable carrier;

the relative amounts of such conjugate and such saponin being effective to stimulate or enhance antibody production in a subject,
wherein in the conjugate the ganglioside derivative is [conjugated] covalently bound to Keyhole Limpet Hemocyanin through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative to the ε-aminolysyl group of Keyhole Limpet Hemocyanin, wherein the C-4 carbon is present in a CH_2 group. -

--111. (twice amended) A method of stimulating or enhancing antibody production in a subject which comprises administering to the subject an effective amount of a composition which comprises:

a) a conjugate of i) a GM2 [or GD2] ganglioside derivative which comprises an unaltered oligosaccharide part and an altered ceramide portion comprising a sphingosine base, to ii) Keyhole Limpet Hemocyanin, comprising an ε-aminolysyl group;

b) a saponin derivable from the bark of a Quillaja saponaria Molina tree; and

c) a pharmaceutically acceptable carrier;

the relative amounts of such conjugate and such saponin being effective to stimulate or enhance antibody production in the subject,
wherein in the conjugate the ganglioside derivative is [conjugated] covalently bound to Keyhole Limpet Hemocyanin through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative to the ε-aminolysyl group of Keyhole Limpet Hemocyanin, wherein the C 4 carbon is present in a CH_2 group, so as to thereby stimulate or enhance antibody production in the subject.--

--113. (Twice amended) A method of treating a cancer in a subject which comprises administering to the subject an effective cancer treating amount of a composition which comprises:

a) a conjugate of i) a GM2 [or GD2] ganglioside derivative which comprises an unaltered oligosaccharide part and an altered ceramide

portion comprising a sphingosine base, to ii) Keyhole Limpet Hemocyanin, comprising an ε-aminolysyl group;

b) a saponin derivable from the bark of a Quillaja saponaria Molina tree; and

c) a pharmaceutically acceptable carrier;

the relative amounts of such conjugate and such saponin being effective to stimulate or enhance antibody production in the subject,
wherein in the conjugate the ganglioside derivative is [conjugated] covalently bound to Keyhole Limpet Hemocyanin through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative to the ε-aminolysyl group of Keyhole Limpet Hemocyanin, wherein the C-4 carbon is present in a CH_2 group, so as to thereby treat the cancer in the subject.--

Exhibit B

Ganglioside Vaccines With Emphasis on GM2

Philip Livingston

Gangliosides are neuraminic acid–containing glycosphingolipids that are anchored into the cell membrane lipid bilayer by lipophilic ceramide chains. They are overexpressed on tissues of neuroectodermal origin, and particularly in tumors such as melanomas, sarcomas, neuroblastomas, astrocytomas, and small cell lung cancers. Both active and passive immunotherapy trials have identified gangliosides as uniquely effective targets for antibody mediated melanoma immunotherapy. Induction of antibodies against GM2 by vaccination has correlated with an improved prognosis in American Joint Committee on Cancer (AJCC) stage III melanoma patients and vaccines containing GM2 chemically conjugated to keyhole limpet hemocyanin (KLH: GM2-KLH) plus the immunologic adjuvant QS-21 have proven to be consistently immunogenic. Phase III trials with this vaccine are ongoing in patients with melanoma in the United States, Canada, Europe, Australia, and New Zealand. GD2, fucosylated GM1, and GD3-KLH conjugates plus QS-21 are also consistently immunogenic, inducing IgM and IgG antibodies in the majority of patients. Polyvalent ganglioside-KLH conjugate plus QS-21 vaccines should be available in early 1999 for testing in phase II and III clinical trials.
Semin Oncol 25:636-645.

IN 1975, Drs Lloyd Old, Herbert Oettgen, and I initiated a series of immunization trials with melanoma and melanoma lysate vaccines mixed with various adjuvants. One hundred ten patients were immunized and the resulting serologic, delayed-type hypersensitivity (DTH), and (in the 24 patients in whom autologous melanoma cell lines were available) cytotoxic T lymphocyte (CTL) responses analyzed.[1-6] While high levels of cell-mediated cytotoxicity were detected against cultured autologous melanoma cells in some of these patients, including what was subsequently identified as human leukocyte antigen (HLA)-A2–restricted reactivity against tyrosinase in one patient, these reactivities were present before immunization.[6,7] The immunizations had no impact on cytotoxic T-cell reactivity. Vaccine-induced DTH reactions were not interpretable, since specificity could not be analyzed definitively. Serologic responses against melanoma antigens on autologous and allogeneic melanoma cells were detected in 11 patients. After extensive specificity analysis, the only antigens recognized by more than one patient were the gangliosides GM2 and GD2. Tai et al[8] also found GM2 and GD2 to be uniquely immunogenic. Ten of 26 patients vaccinated with a mix of irradiated allogeneic melanoma cell lines produced IgM antibodies against GM2 and two patients produced antibodies against GD2. Gangliosides have also been shown to be effective targets for passive immunotherapy of melanoma with monoclonal antibodies. Major clinical responses have resulted from treatment of patients with monoclonal antibodies against GM2, GD2, and GD3.[9-15] Hence, both active and passive immunotherapy trials have identified gangliosides as uniquely effective targets for melanoma immunotherapy.

Gangliosides are neuraminic acid–containing glycosphingolipids that are anchored into the lipid bilayer of the plasma membrane by their lipophilic ceramide moiety. The carbohydrate portions of gangliosides are present on the extracellular border of the plasma membrane, where they are available for recognition by antibodies. The structures of the gangliosides discussed in this review and the close proximity of the immunogenic carbohydrate epitopes to the cell membrane are demonstrated in Fig 1.

THE BASIS FOR VACCINES THAT INDUCE ANTIBODIES

Antibodies are the primary mechanism for eliminating infectious pathogens from the bloodstream. They are also ideally suited for elimination of circulating tumor cells and micrometastases. The importance of antibodies in mediating protection from tumor recurrence is well documented in experimental animals. Experiments involving administration of monoclonal antibody 3F8 against GD2 are a case in point.[16] Administration of 3F8 before intravenous tumor challenge or as late as 4 days after tumor challenge results in complete

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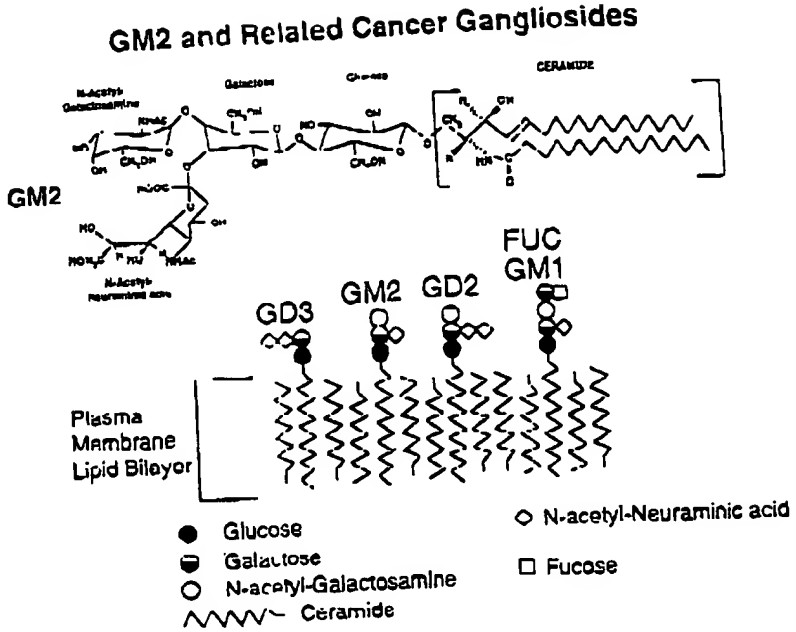

Fig 1. Demonstration of the structure of gangliosides GM2, GD2, GD3, and fucosyl GM1, and their close proximity to the external surface of the cell membrane, where they are anchored by incorporation of ceramide into the cell membrane lipid bilayer.

protection of a majority of mice. This timing may be comparable to antibody induction in or administration to patients in the adjuvant setting, after surgical resection of the primary or lymph node metastases in cancers such as melanoma and after response to chemotherapy in cancers such as small cell lung cancer (SCLC), since in both cases the targets may be circulating tumor cells and micrometastases. Administration of 3F8 seven or more days after tumor challenge had little impact on tumor progression.

There is also evidence in cancer patients that natural or passively administered antibodies in the adjuvant setting are associated with a more favorable prognosis.

(1) Natural antibodies (antibodies present in patient sera before vaccination) have been correlated with an improved prognosis. This is true for patients with paraneoplastic syndromes, in which high titers of antibodies against onconeural antigens expressed on particular cells in the nervous system and certain types of tumors have been associated both with debilitating autoimmune neurologic disorders and with delayed tumor progression and prolonged survival.[17] Also, patients with American Joint Committee on Cancer (AJCC) stage III melanoma and natural antibodies against GM2 ganglioside treated at two different medical centers have had an 80% to 100% 5-year survival rate compared with the expected rate of 40%,[18,19] as shown in Fig 2.

(2) Tumor vaccine-induced antibodies against GM2 (see Fig 2) and several other melanoma antigens at four different medical centers, and against sialyl Tn antigen in adenocarcinoma patients, have correlated with prolonged disease-free interval and survival.[19-23]

(3) Patients with Dukes C colon cancer treated with monoclonal antibody 17-1A in the adjuvant setting had a significantly prolonged disease-free and overall survival compared with randomized controls.[24]

Hence, in the adjuvant setting, passively administered and vaccine-induced antibodies have been shown to correlate with improved disease-free and overall survival in the mouse and in humans. Since the great majority of cancer patients are initially rendered free of detectable disease by surgery and or chemotherapy after initial diagnosis, vaccines that induce antibodies may have broad applicability.

EXPRESSION OF GANGLIOSIDES AT THE CELL SURFACE OF CANCERS AND NORMAL TISSUES

Ganglioside expression in a variety of malignancies has been documented by extraction, followed by thin-layer chromatography and immune thin-

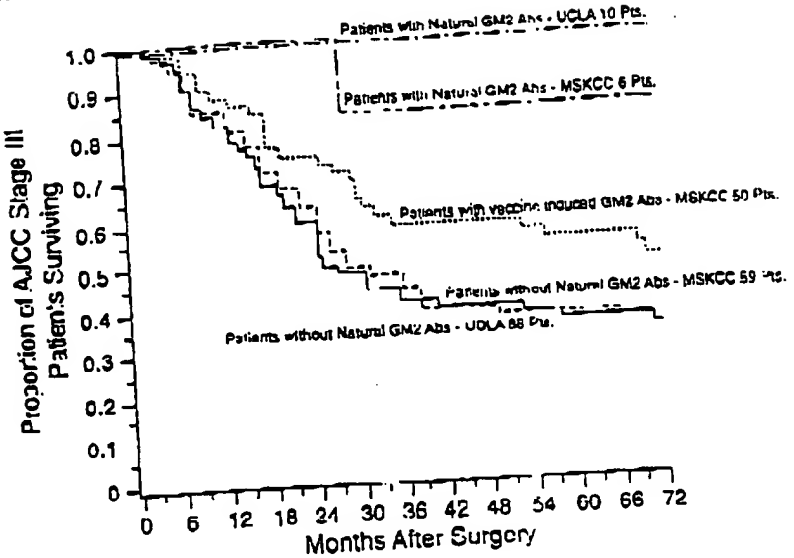

Fig 2. Correlation between the presence of natural or GM2/BCG vaccine-induced serum antibodies against GM2 and survival in AJCC stage III melanoma patients seen at the Memorial Sloan-Kettering Cancer Center (MSKCC)[11] or the UCLA School of Medicine.[12]

layer chromatography (TLC), and by immunohistology.[25-28] These results are summarized in Table 1. GM2, GD2, and GD3, gangliosides are broadly expressed in melanomas, sarcomas, and neuroblastomas, as well as in a variety of primary brain tumors. Fucosyl GM1 expression has been largely restricted to SCLC.[27,29] 9-O-acetyl GD3 (GD3 O-acetylated at the 9 position of the terminal sialic acid) has been widely expressed only in melanomas. Surprisingly, GM2 also appears to be expressed in the majority of epithelial cancers and may represent a broadly expressed antigen.

Table 1. Expression of Gangliosides Detected by Extraction and TLC, or by Immunohistology in >75% of Human Cancer Biopsy Specimens

Tumor	Extraction	Immunohistology	References
Melanoma	GD3, GD2, GM2, 9-O-acetyl GD3	GM2, GD3, GD2	25, 27, 63
Sarcoma	GD2, GM2, GD3	GM2, GD2, GD3	27, 59
Neuroblastoma	GD2, GM2, GD3	GM2, GD2, GD3	27, 30, 59, 60
1° brain tumors	GD2, GD3, GM2		59, 60
SCLC	FucGM1, GD2, GD3	GM2, FucGM1	27, 29, 59, 61
Epithelial cancers	GM2	GM2	27, 62

Gangliosides are also present on a variety of normal tissues.[27] GM2, GD2, and GD3 are expressed on brain cells, especially GD2, which is also expressed on some peripheral nerves. Unexpectedly, GD2 was found to be present on some B lymphocytes in the spleen and lymph nodes (but not in peripheral blood) and GM2 was detected at the secretory borders of most epithelial tissues. GD2 and GD3 were also expressed, though at lower levels, in connective tissues of multiple organs, and GD3 is known to be expressed on a subset of human T lymphocytes.[30] Fucosyl GM1 was seen in only occasional cells in the islets of Langerhans and some sensory neurons in the dorsal root ganglia.

There is now sufficient experience from clinical trials with vaccine-induced antibody responses against GM2, GD2, and several other nonganglioside antigens, and passive administration of monoclonal antibodies against GD2, GD3, and several other antigens to draw conclusions about the consequences of antigen distribution on various normal tissues. GM2, GD2, and GD3 exposure on cells in the brain and GM2 and sTn expression in cells at the secretory borders of epithelial tissues induce neither immunologic tolerance nor autoimmunity once antibodies are present. This suggests that in each case they are sequestered from the immune system. Treatment of patients with monoclonal antibodies against GD2 and GD3 has not induced CNS toxicity in children or adults. Induc-

tion of antibodies against GM2 and other antigens expressed at the secretory borders of epithelial tissues such as sTn and TF disaccharides[31-34] has not induced detectable toxicity. On the other hand, high doses of some monoclonal antibodies against GD2 have induced significant neuropathies as a consequence of GD2 expression on peripheral nerves.[35] Administration of a monoclonal antibody against Lewis X (which is expressed at the secretory borders of several epithelial tissues and also on circulating polymorphonucleosites) has resulted in no toxicity related to the epithelial tissue expression, but profound, though short-lived, neutropenia after each administration.[35,36] These two examples demonstrate that antibodies against antigens that are not sequestered from the immune system can have profound effects. Against this background, GM2, GD3, 9-O-acetyl GD3, and fucosyl GM1 all appear to be excellent targets for active immunotherapy with vaccines. Since peripheral neuropathy developed with only some monoclonal antibodies against GD2, but not others, and only at the higher doses, it may be that GD2 can also serve as a safe target for vaccine-induced antibodies.

MECHANISMS OF ANTIBODY ACTION

In general, interaction of antibody and antigen is without significance unless Fc-mediated secondary effector mechanisms are activated. On the basis of studies of bacterial infections, the most important mechanism of protection by antibodies is complement-mediated attack and lysis. IgM antibody bound to cell-surface carbohydrate antigens such as gangliosides is the most active complement activator in the intravascular space,[37,38] while IgG1 and IgG3 may be the most important extravascularly. Complement activation at the cell surface mediates inflammatory reactions, opsonification for phagocytosis, clearance of antigen-antibody complexes from the circulation, and membrane-attack complex-mediated lysis. Receptors on IgG1 and IgG3 are also the primary targets for effector cells mediating antibody-dependent cell-mediated cytotoxicity (ADCC) of tumor cells. FCγR1 (CD64), FCγR2 (CD32), and FCγR3 (CD16) receptors on a range of effector cells, including especially natural killer cells, but also T lymphocytes and cells of myeloid lineage, react with tumor cell–bound antibodies, resulting in activation of inherent cytotoxic mechanisms in the effector cells.

If antibodies of sufficient titer can be induced against cell-surface antigens to eliminate tumor cells from the blood and lymphatic systems and to eradicate micrometastases, as demonstrated in mice with antibodies against GD2,[16] this would dramatically change our approach to treating the cancer patient. With repeated showers of metastases no longer possible as a consequence of high levels of circulating antibodies, aggressive local therapies of established metastases (surgery, radiation therapy and intralesional injections) might result in long-term control of even metastatic cancers. It is also possible that complement-mediated inflammation, improved antigen presentation by specifically immune B lymphocytes, and decreased circulating tumor antigen may facilitate T-lymphocyte immunity, as has been demonstrated in other systems.[39-41]

IMMUNOGENICITY OF EARLY GM2 GANGLIOSIDE VACCINES

Initial clinical studies with whole melanoma cell vaccines demonstrating the relatively high immunogenicity of GM2 ganglioside, and the availability of purified GM2 ganglioside, were the basis for conducting a series of small clinical trials using purified GM2 for vaccine production. Serologic response against purified GM2 and tumor cells expressing GM2 was the end point.[3] Enzyme-linked immunosorbent assay (ELISA) results are summarized in Table 2. In initial trials, GM2 mixed with or adherent to the surface of various bacteria, liposomes, or proteosomes was significantly more immunogenic than GM2 alone. Of these, the proteosome and BCG vaccines were most immunogenic, inducing IgM antibodies in the majority of patients and IgG antibodies in occasional patients. Proteosome is the term used to describe preparations of the highly hydrophobic outer membrane protein (OMP) of *Neisseria meningitidis*, which naturally form liposome-like multimolecular vesicular structures that readily incorporate antigens containing hydrophobic anchor moieties such as gangliosides.[42] In these studies, it appeared that pretreatment of patients with low-dose cyclophosphamide intravenously (300 mg/m^2), which was intended to decrease suppressor-cell activity, resulted in increased antibody titers against GM2. Overall, BCG was the most effective adjuvant. Moderate-titer IgM antibodies were induced in the majority of patients and low-titer IgG antibodies

Table 1. Peak GM2 Antibody Titer After Adjuvant Immunization of Stage III/IV Melanoma Patients With Vaccines Containing Purified $GM2^{Mimetic}$

Vaccine	Total Patients Treated	ELISA IgM Patients With Antibodies	ELISA IgM Median Titer	ELISA IgG Patients With Antibodies	ELISA IgG Median Titer
GM2	5	0	0	0	0
GM2/R595	5	0	0	0	0
CY + GM2/R595	6	5	1/40	0	0
GM2/MPLA liposomes	6	1	1/40	0	0
GM2/proteosomes	22	22	1/160	4	1/40
GM2/BCG	5	4	1/120	0	0
CY + GM2/BCG	58	50	1/160	6	1/80
CY + GM2-KLH	6	5	1/80	0	0
CY + GM2-KLH/BCG	6	4	1/240	1	1/320
CY + GM2-KLH/Detox	6	5	1/160	0	0
CY + GM2-KLH/QS-21	9	9	1/640	8	1/160
GM2-KLH/QS-21	40	39	1/640	35	1/160

Abbreviations: ELISA, enzyme-linked immunosorbent assay; MPLA, monophosphoryl lipid A; BCG, bacillus Calmette Guérin; KLH, keyhole limpet hemocyanin; CY, cyclophosphamide; R595, *Salmonella minnesota* mutant R595; Detox, mixture of MPLA and BCG cell-wall skeletons; QS21, purified fraction from Quillaja saponaria bark; proteosomes, liposome-like vesicles formed from hydrophobic Neisseria meningitidis outer-membrane proteins.

were induced in occasional patients. Antibody titers in most patients returned to baseline within 8 to 10 weeks after each immunization, and even with subsequent booster immunizations this pattern of antibody reactivity and duration did not change. This is consistent with GM2 acting as a T-cell independent antigen. There was a suggestion from these initial studies that melanoma recurrence was delayed in patients developing GM2 antibody titers of ≥1/40, regardless of the adjuvant used.[34]

The expression of GM2 on most melanomas, the consistent IgM antibodies induced in patients immunized with the GM2/BCG vaccine, and the correlation of induction of GM2 antibody titers with a more favorable prognosis[34] provided the rationale for conducting a randomized trial to determine whether clinical benefit would result from vaccine-induced GM2 antibody production.[19] One hundred twenty eligible AJCC stage III melanoma patients who were free of disease after surgery were randomized to receive GM2/BCG vaccine or to receive BCG alone. All patients were pretreated with low-dose cyclophosphamide. With a minimum follow-up duration of 72 months, there was a 23% increase in the disease-free interval ($P = .004$) and a 17% increase in overall survival ($P = .03$) in patients who produced antibody titers against GM2 of 1/40 or more compared with antibody-negative patients, confirming our earlier experience (Fig 2). Comparing the treatment (GM2/BCG) and control (BCG) groups and excluding the six patients with preexisting GM2 antibodies from statistical analysis (one in the GM2/BCG group and five in the BCG group) resulted in a 17% increase in disease-free interval ($P = .02$) and a 14% increase in overall survival ($P = .15$) for patients with the GM2/BCG vaccine. However, when all patients in the two treatment groups were compared as randomized, these increases were 14% for disease-free interval and 11% for survival in the GM2/BCG treatment group, with neither result achieving statistical significance.

Although these results were encouraging, there was room for improvement. The IgM antibodies induced were of only moderate titer and short-lived. In addition, only occasional IgG antibodies against GM2 were induced. To improve the immunogenicity of the vaccine, we pursued two major lines of endeavor. Initially, we made modifications in the ganglioside structure. This was intended to permit the gangliosides to be recognized as foreign and so result in higher titer antibodies that would cross react with the original unmodified ganglioside.[46,47] After an extensive series of trials, this

approach was abandoned, because though high titer antibodies were indeed induced against the modified gangliosides, there was no cross reactivity with the unmodified ganglioside. The second approach was to augment helper T-cell reactivity and antigen processing by chemically conjugating the ganglioside to an immunogenic carrier protein and using a more potent immunologic adjuvant.

THE GM2-KEYHOLE LIMPET HEMOCYANIN CONJUGATE VACCINE PLUS QS21

Following the lead of bacterial polysaccaride vaccines that had shown that covalent attachment of antigens to immunogenic carrier proteins resulted in the highest titer antibody responses, we explored the use of ganglioside conjugate vaccines ± immunologic adjuvants.[48] Keyhole limpet hemocyanin (KLH) was the best of the six immunogenic carrier molecules tested in the mouse, the method of conjugation was crucial, and a potent immunologic adjuvant was required. GD3 conjugated via the ceramide moiety (not the carbohydrate moiety) of the ganglioside and mixed with immunologic adjuvant QS21 was optimal. QS21 is a purified homogenous saponin fraction obtained from the bark of the *Quillaja saponaria* Molina tree.[49] Simple mixture of GD3, KLH, and QS21 induced no antibodies. A variety of different carriers and adjuvants have also been tested with gangliosides GM2, GD2, and fucosyl GM1.[43,56] In each case, the ganglioside covalently attached to KLH via the ceramide moiety plus QS21 induced the highest titers of IgM and IgG antibodies. Consequently, this is the approach applied to subsequent clinical trials.

Results of initial clinical trials with the GM2-KLH conjugate vaccine plus various adjuvants are summarized in Table 2.[3] Pretreatment with cyclophosphamide had no impact on the antibody titers induced by the GM2-KLH conjugate plus QS21 vaccine. In subsequent studies, GM2 doses of 3, 10, 30 and 70 μg per vaccine were tested and the 30-μg dose selected for all future trials. In addition, it appeared that GM2-KLH epitope ratios in the conjugate of greater than 600/1 were more consistently immunogenic than lower ratios. The induced antibodies were shown to be highly specific for GM2 with minimal cross-reactivity directed against GD2 and GM3 and no cross-reactivity against other gangliosides.[43] IgG antibodies induced in immunized patients were of the IgG subclasses IgG1 and IgG3. Both IgM and IgG antibodies in most patients were able to activate complement-mediated lysis of GM2-positive, but not GM2-negative, tumor cells, and IgG antibodies from most patients were able to mediate ADCC.[50] The median duration of antibody titers of 1/40 or greater induced by the GM2-KLH plus QS21 vaccine was 6 months. Consequently, this conjugate vaccine was a clear improvement (in terms of antibodies induced) compared with the previous GM2/BCG vaccine. This provided the basis for initiating phase III clinical trials aimed at demonstrating the impact of vaccination with the GM2-KLH plus QS21 vaccine on disease-free and overall survival.

In the United States, a randomized phase III adjuvant trial comparing high-dose interferon-alfa versus the GM2-KLH plus QS21 vaccine is being conducted in patients with deep AJCC stage II primary melanomas (>4 mm depth) or stage III disease (positive regional lymph nodes) by the Eastern Cooperative Oncology Group, the Southwest Oncology Group, the North Central Cancer Treatment Group, Cancer and Leukemia Group B, Memorial Sloan-Kettering Cancer Center (MSKCC), and M.D. Anderson Cancer Center. In Europe, New Zealand, and Australia, the same patient group will be randomized to receive the same GM2 vaccine or placebo. A trial in AJCC stage II patients with thin primary tumors (2 to 4 mm) will be initiated in Europe and the United States in 1999.

IMMUNOGENECITY OF OTHER GANGLIOSIDES

GD2, GD3 and 9-O-Acetyl GD3

Using BCG, as adjuvant, we have immunized melanoma patients with GM2, GD2, GD3, GD3 lactone, and a series of O-acetyl GD3 gangliosides. The results are summarized in Table 3. GD3 was not found to be immunogenic in any patient. GD2 and GD3 lactone were found to be immunogenic in occasional patients, suggesting that with a more effective immunization approach they too might be consistently immunogenic. The O-acetyl GD3 vaccines induced antibodies that reacted with the immunizing gangliosides, but not melanoma O-acetyl GD3, which is thought to be acetylated at the 9-O position of the terminal carbon.[51] Nuclear magnetic resonance (NMR) analysis demonstrated that none of the O-acetylated GD3 preparations

Table 3. Relative Immunogenicity of Gangliosides GM2, GD2, GD3, GD3 Lactone, 9-O-Acetyl GD3, and Fucosyl GM1 in Patients Immunized With Ganglioside/BCG or Ganglioside-KLH Plus QS21 Vaccines

Vaccine	GM2		GD2		GD3		GD3 Lactone		9-O-Acetyl GD3		Fucosyl GM1	
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
Ganglioside/BCG												
Patients vaccinated	22		12		12		9		12			
Patients making antibodies	70	16	3	0	0	0	4	0	0	0		
Median peak titer	160	80	40	0	0	0	40	0	0	0		
Ganglioside-KLH + QS21												
Patients vaccinated	30		6		7		6		6		11	
Patients making antibodies	30	24	6	0	2	0	3	5	5	5	11	10
Median peak titer	960	160	160	0	40	0	160	320	640	640	640	640

used for vaccine construction were acetylated exclusively or even primarily at the 9-O position.

We have recently immunized small groups of patients with GD3-KLH and GD3 lactone-KLH conjugate vaccines mixed with QS21. The results are summarized in Table 3. Once again, GD3 proved nonimmunogenic, but GD3 lactone succeeded in inducing antibodies against GD3, GD3 lactone, and melanoma cells expressing GD3 in the majority of patients. The basis for the increased immunogenicity of GD3 lactone over GD3 for inducing antibodies against GD3 may be the increased rigidity of GD3 lactone molecules, which is thought to increase immunogenicity, as previously described for GM3 lactone.[52] Antibodies against GD3 have also been induced in some patients by vaccination with the antiidiotype monoclonal antibody BEC2.[53] Chapman has described the induction of antibodies against GD3 in up to 30% of patients vaccinated with BEC2, depending on the adjuvant used and the route of administration.[53,54]

Trials with GD2-KLH vaccines have also succeeded in inducing antibodies detectable against synthetic GD2 by ELISA,[55] against tumor biopsy specimens by immune thin layer chromatography, and against GD2-positive cultured tumor cells by flow cytometry in the majority of patients. Analysis of these trials is still ongoing, but it is already clear that the antibodies from the majority of GD2 and GD3 lactone–vaccinated patients react with melanoma cells expressing the same antigens.

Fucosyl GM1

Eleven patients with SCLC who were free of grossly detectable disease after response to multiple cycles of chemotherapy were immunized with a fucosyl GM1-KLH plus QS21 vaccine.[56] All patients produced high-titer IgM antibodies (median titer, 1/1,280) and 10 produced moderate-titer IgG antibodies (median titer, 1/640) against fucosyl GM1. Antibodies were also reactive with SCLC biopsy-derived fucosyl GM1 by immune thin-layer chromatography, and with SCLC cell lines expressing fucosyl GM1 by flow cytometry. These results are summarized in Table 3.

FUTURE DIRECTIONS FOR GANGLIOSIDE VACCINES

While there is every indication that immunization with these single antigens may prove beneficial when administered in the adjuvant or minimal disease setting, in the long run polyvalent vaccines offer the most promise. This is because functional and antigenic heterogeneity are inherent features of malignancies and genetically based heterogeneity of responsiveness is inherent in the human immune response. Only with polyvalent vaccines can we hope to induce an immune response capable of eliminating every cancer cell. We have immunized groups of mice with mixtures of four of the individual KLH conjugate vaccines, which were either injected as a mixture in a single syringe or as individual vaccines administered to four quadrants in the same mouse, and compared immune responses to the response obtained when mice were immunized with a single one of these four components. No loss of immunogenicity was detected when four antigens were injected to the same mouse, and use of a single syringe with the four vaccines mixed together was as effective at inducing high-titer IgM and IgG antibodies against each of the peptide and carbohydrate antigens, as were the other alternatives.

We can now consistently induce IgM antibodies and in most cases IgG antibodies against GM2, GD2, and fucosyl GM1. In all cases, the antibodies react not only with the synthetic or purified immunizing ganglioside, but also with the same ganglioside obtained from tumor specimens and with cultured tumor cells. The GD3 lactone-KLH vaccine may also be ready to add to this list, but our experience with it has been quite limited. An additional GD3 lactone-KLH trial has recently been initiated to confirm our previous results. Also, we are preparing to compare the immunogenicity of GD3 lactone-KLH, with the antiidiotype monoclonal antibody BEC2 vaccine, and combinations of these two vaccines, to determine whether the combination is able to induce more consistent antibodies against GD3 and GD3-positive tumor cells than GD3 lactone-KLH or BEC2 alone.

By mid 1999, we anticipate combining the optimal single antigen vaccines described above into polyvalent vaccines. The antigens known to be expressed by different cancers as determined by extraction and immunohistology (Table 1) will guide vaccine construction. Vaccines against melanoma, neuroblastoma, and primary brain cancers will contain only the ganglioside antigens indicated, since we are not aware of any other well-defined tumor antigens that are expressed at the cell surface and are available for vaccine construction. Vaccines against neuroblastoma and SCLC on the other hand will contain the three gangliosides indicated in Table 1 in each case, but also one or more nonganglioside antigens that are known to be expressed at the cell surface of these cancers (ie, polysialic acid for neuroblastoma and polysialic acid, Globo H, and KSA for SCLC) as previously described.[27,57,58] Vaccines against epithelial cancers will contain GM2 as the only ganglioside and a variety of other carbohydrate and peptide antigens known to be expressed at the cell surface.[57,58] Once these pilot trials have demonstrated the safety and immunogenicity of these polyvalent vaccines, randomized phase III trials in the adjuvant setting will follow.

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Exhibit C

accines for Melanoma: Superior Immunogenicity of Keyhole Limpet cyanin Conjugate Vaccines[1]

hn Helling,[2] Ann Shang, Michele Calves, Shengle Zhang, Shunlin Ren, Robert K. Yu, Herbert F. Oettgen, and). Livingston

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ACT

urface gangliosides show altered patterns of expression as a con- : of malignant transformation and have therefore been of interest itial targets for immunotherapy, including vaccine construction. tacle has been that some of the gangliosides that are overexpressed in cancers are poorly immunogenic in humans. A case in point is prominent ganglioside of human malignant melanoma. Using an ch that has been effective in the construction of bacterial carbo- : vaccines, we have succeeded in increasing the immunogenicity of the mouse by conjugating the ganglioside with immunogenic car- several conjugation methods were used. The optimal procedure d ozone cleavage of the double bond of G_{D3} in the ceramide back- ntroducing an aldehyde group, and coupling to aminolysyl groups teins by reductive amination. Conjugates were constructed with a ic multiple antigenic peptide expressing repeats of a malarial T-cell e, outer membrane proteins of *Neisseria meningitidis*, cationized : serum albumin, keyhole limpet hemocyanin, and polylysine. Mice nized with these conjugates showed a stronger antibody response to nan mice immunized with unconjugated G_{D3}. The strongest response bserved in mice immunized with the keyhole limpet hemocyanin gate of the G_{D3} aldehydic derivative and the adjuvant QS-21. These showed not only a long-lasting high-titer IgM response but also a stent high-titer IgG response (predominantly IgG1), indicating re ment of T-cell help, although the titers of IgM and IgG antibodies wing booster immunizations were not as high as they are in the onse to classical T-cell-dependent antigens. This method is applicable ther gangliosides, and it may be useful in the construction of immu- nic ganglioside vaccines for the immunotherapy of human cancers ressing gangliosides on their cell surface.

RODUCTION

Gangliosides are glycolipid constituents of the cell membrane. The n was coined in 1942 to refer to lipids of the central nervous em that contained sialic acid, to signify their prime location in glion cells and their glycosidic nature (1). Their lipophilic com- ent, the ceramide (an amide linked long-chain sphinguid base and tty acid), is thought to be embedded in the outer membrane of the membrane lipid bilayer. The carbohydrate portion of the molecule riented toward the outside of the cell. Malignant transformation ears to activate enzymes involved in ganglioside glycosylation, ulting in altered patterns of ganglioside expression in tumors such astrocytoma, neuroblastoma, and malignant melanoma (2). In nor- l melanocytes, for example, the predominant ganglioside is G_{M3}.[3] er gangliosides including G_{D3}, G_{M2}, G_{D1a}, and G_{T1b} constitute s than 10% of the total (3). In malignant melanoma, increased expression of G_{D3}, G_{D2}, and G_{M2} has been observed (4, 5), and these gangliosides have therefore been considered potential targets for immunotherapy.

One approach to ganglioside-targeted immunotherapy has been the use of mAbs.[4] Treatment of patients with melanoma or neuroblastoma with mAb recognizing G_{D3}, G_{D2}, or G_{M2} has resulted in tumor regression in some cases (6–9). The other approach has been to immunize patients with ganglioside vaccines in attempts to induce production of ganglioside antibodies by the patients themselves. These attempts have been successful so far only with G_{M2} vaccines. Patients with American Joint Committee on Cancer Stage III malignant melanoma, after complete resection of all tumor, have been shown to produce anti-G_{M2} antibodies in response to vaccination with G_{M2} and *Bacillus Calmette-Guérin* (after pretreatment with low-dose cyclophosphamide to reduce suppressor activity), and the disease-free interval and overall survival were longer in patients producing G_{M2} antibodies (10). G_{D3} and G_{D2}, on the other hand, were found to be only rarely immunogenic when administered in the same way to patients with melanoma (11). Even with the G_{M2} vaccines, the antibody response showed the characteristics of a T-cell-independent response, that is to say, IgM production of short duration, rare conversion to IgG production, and lack of a booster effect (12, 13).

Similar difficulties have been encountered in the development of effective vaccines against bacterial carbohydrate antigens. One approach that has been successful in overcoming these problems is conjugation of the antigen with immunogenic protein carriers. For example, a conjugate vaccine that links the *Haemophilus influenzae* type b capsular polysaccharide to the outer-membrane protein complex of *Neisseria meningitidis* serogroup B was recently shown to induce the production of antibodies and a high rate of protection against invasive disease caused by *Haemophilus influenzae* type b in infants (14), and similar results were reported for a conjugate vaccine using a nontoxic mutant diphtheria toxin as carrier (15).

We have explored this approach in attempts to increase the immunogenicity of melanoma gangliosides. We report here the effects of conjugating G_{D3} with several protein carriers on its immunogenicity in the mouse.

MATERIALS AND METHODS

Gangliosides. G_{M3}, G_{M2} and G_{D1a}, extracted from bovine brain, were provided by Fidia Research Laboratory (Abano Terme, Italy). G_{D2} was made from G_{D1a} by enzymatic cleavage with β-galactosidase from bovine testes (16). G_{D3} (mel) was isolated from human melanoma tissue (17), G_{D3} (bbm) and $GT3$ were isolated from bovine buttermilk (18), and disialyllactose ($GD3$ oligosaccharide) was isolated from bovine colostrum as previously described (19).

Reagents. HPTLC silica gel plates were obtained from E. Merck (Darmstadt, Germany); 4-chloro-1-naphthol, *p*-nitrophenyl phosphate disodium, and sodium cyanoborohydride were from Sigma Chemical Co. (St. Louis, MO);

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The designations G_{M3}, G_{M2}, G_{D3}, G_{D2}, G_{D1a}, and G_{D1b} are used in accordance with abbreviated ganglioside nomenclature proposed by Svennerholm (40).

The abbreviations used are: mAb, monoclonal antibody; MAP, multiple antigenic peptide; OMP, outer membrane protein; cBSA, cationized bovine serum albumin; ITLC, immune thin-layer chromatography; HPTLC, high-performance thin-layer chromatography; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; bbm, bovine buttermilk.

methylsulfide was from Aldrich (Milwaukee, WI); cyclophosphamide (Cytoxan) was from Mead Johnson (Syracuse, NY); and QS-21 adjuvant, a heterogeneous saponin component purified from *Quillaja saponaria* Molina tree (20), was kindly donated by Cambridge Biotech Corp. (Worcester, MA). It is an amphipathic molecule and was provided as a white powder, forming a clear colorless solution when dissolved in PBS.

Proteins. Poly-L-lysine hydrobromide [MW(vis)3800] was purchased from Sigma, keyhole limpet hemocyanin (KLH) was from Calbiochem (La Jolla, CA), the eBSA-Imject Supercarrier immune modulator was from Pierce (Rockford, IL), and *Neisseria meningitidis* OMPs were kindly provided by Dr. M. S. Blake (Rockefeller University, New York). MAP YAL-IV 294-1 containing four repeats of a malarial T-cell epitope was a gift from Dr. J. P. Tam (Rockefeller University).

Monoclonal Antibodies. Rabbit anti-mouse immunoglobulins conjugated to horseradish peroxidase for HTLC, and rabbit anti-mouse IgM and IgG conjugated to alkaline phosphatase for ELISAs, were obtained from Zymed (San Francisco, CA); anti-G_{D3} mAb R24 was generated in our laboratory (21).

Serological Assays. ELISA were performed as previously described (13). To control for nonspecific "stickiness," immune sera were also tested on plates to which no ganglioside had been added, and the reading was subtracted from the value obtained in the presence of ganglioside. The titer was defined as the highest dilution yielding a corrected absorbance of 0.1 or greater. Immunostaining of gangliosides with mAb or mouse sera was performed after separation on HPTLC silica gel glass plates as previously described (4). Plates were developed in solvent 1 [chloroform:methanol:water (0.25% $CaCl_2$), 50:40:10 (v/v)] or solvent 2 [ethanol:n-butylalcohol:pyridine:water:acetic acid, 100:10:10:30:3 (v/v)], and gangliosides were visualized with resorcinol-HCl reagent. Dot-blot immune stains were performed on nitrocellulose strips utilizing purified gangliosides spotted in equal amounts and developed as described before (13).

Immunization. Six-week-old female BALB/c × C57BL/6 F_1 mice (The Jackson Laboratory, Bar Harbor, ME) were given an i.p. injection of cyclophosphamide (15 mg/kg) 3 days before the first immunization and were then assigned to treatment groups. Groups of 4 or 5 mice were given three s.c. injections of a vaccine 2 weeks apart if not otherwise indicated. Each vaccine contained 20 μg G_{D3} or 15 μg disialyllactose and 10 μg QS-21 in a total volume of 0.1 ml PBS. Mice were bled from the retroorbital sinus before vaccination and 2 weeks after the last vaccine injection unless indicated otherwise.

G_{D3} Conjugate Preparation. G_{D3} (2 mg) was dissolved in 2 ml methanol by sonication and cooled to −78°C in an ethanol/dry ice bath. Ozone was generated in an ozone generator (Del Industries, San Luis Obispo, CA) and was passed through the sample for 30 min under vigorous stirring (22, 23). The excess of ozone was then displaced with nitrogen over a period of 10 min. Methylsulfide (100 μl) was added (24), and the sample was kept at −78°C for 30 min and then at room temperature for 90 min under vigorous stirring. The sample was dried under a stream of nitrogen and monitored by HPTLC. The long-chain aldehyde was separated by adding *n*-hexane (2 ml) to the dry sample, followed by sonication for 5 min and centrifugation at 2000 × *g* for 15 min. The *n*-hexane was carefully drawn off and discarded, and the sample was dried under a stream of nitrogen. Cleaved G_{D3} and native G_{D3} were separated by HPLC (Waters, System 501, Milford, MA) utilizing a C_{18} reversed-phase column (10 x 250 mm; Rainin Instruments, Ridgefield, NJ). Gangliosides were eluted with a linear water-acetonitrile gradient and monitored at 214 nm, and the fractions were analyzed by HPTLC. Fractions that contained cleaved G_{D3} were combined and evaporated at 37°C with a Rotavapor (Büchi, Flawils, Switzerland). Cleaved G_{D3} (1.5 mg), 1.5 mg protein carrier in PBS, and 2 mg sodium cyanoborohydride were incubated under gentle agitation at 37°C for 48 h. After 16 h 1 mg sodium cyanoborohydride was added. The progress of coupling was monitored by HPTLC. G_{D3}-protein conjugates did not migrate in solvent 1 and solvent 2 but remained at the origin as a resorcinol-positive band. The mixture was dialyzed across 5000 molecular weight cutoff dialysis tubing with three changes of PBS (4 liters each), at 4°C for 48 h, and passed through an Extractigel detergent-removing gel (Pierce, Rockford, IL) for final purification of unconjugated G_{D3}. The samples were lyophilized, and their protein and ganglioside content was determined by BioRad protein assay and by neuraminic acid determination according to the method of Svennerholm (25).

Disialyllactose Conjugate Preparation. Disialyllactose was isolated from bovine colostrum as described previously (19). The carbohydrate was attached to protein by reductive amination (26). Disialyllactose (10 mg) was incubated with 2 mg proteins in 2 ml PBS for 14 days at 37°C after sterile filtration. Sodium cyanoborohydride (2 mg) was added at the beginning, and 1 mg was added every 3 days. The coupling was monitored by HPTLC in solvent 2. The disialyllactose conjugates were purified by dialysis across 5000 molecular weight cutoff dialysis membrane with three changes of PBS (4 liters each) at 4°C for 48 h, followed by lyophilization. The protein and neuraminic acid content was determined as described above. Disialyllactose was also conjugated to proteins according to the method described by Roy and Laferrière (27). During this procedure *N*-acroloylated glycopyranosylamine derivatives of the oligosaccharide were formed first, followed by conjugation via Michael addition to amino groups of the protein. Purification and determination of protein and neuraminic acid content were performed as described above.

Determination of Antibody Subclasses. Determination of antibody subclasses was performed by ELISA using subclass-specific rabbit anti-mouse immunoglobulins IgG1, IgG2a, IgG2b, IgG3, and IgA (Zymed, San Francisco, CA). Alkaline phosphatase-labeled goat anti-rabbit IgG served as the signal-generating reagent.

FACS Analysis of Mouse Antisera. A single cell suspension of the melanoma cell line SK-MEL-28 was obtained after treatment with 0.1% EDTA in PBS followed by passage through a 26½-gauge needle. Cells (3×10^5) were incubated with 40 μl of 1:20 diluted post- or preimmunization serum for 30 min on ice. The cells were washed three times with 3% fetal calf serum in PBS. Thirty μl of diluted (1:50) fluorescein isothiocyanate-labeled goat anti-mouse IgG (Southern Biotechnology Associates Inc., Birmingham, AL) were added as secondary antibody, followed by incubation on ice for 30 min. Cells were washed three times as above and resuspended in 500 μl 3% fetal calf serum in PBS and analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA).

RESULTS

Preparation and Characterization of G_{D3}-Protein Conjugates. G_{D3} (bbm) in methanol was selectively cleaved with ozone at the C4-C5 double bond in the ceramide portion. It is assumed that methoxyperoxides are formed as intermediate products (24), and therefore methylsulfide was added as a reducing agent. The result of the cleavage was a G_{D3} derivative with an aldehyde functional group in the position of the former double bond in the ceramide portion (Fig. 1). Cleaved G_{D3} migrated slower than native G_{D3}, and formed double bands because the ceramide contained unsaturated fatty acids that were cleaved simultaneously (see Fig. 1, *inset*). Densitometric analysis of HPTLC plates showed that more than 70% of G_{D3} (bbm) was cleaved by this procedure. Preliminary experiments involving longer ozone treatment had similar results, indicating that 30% of G_{D3} from this source consists of sphinganine or phytosphingosine analogues that contain no ozone-cleavable ceramide double bond. Cleavage at −78°C with ozone treatment up to 1 h (depending on the amount of G_{D3} used) was found to be optimal. Cleaved G_{D3} persisted only in acidic and neutral phosphate buffers for up to 72 h, but with the formation of increasing amounts of oligosaccharide due to β-elimination reactions [which have been shown to occur much faster at alkaline pH (23)]. The decreased hydrophobicity of cleaved G_{D3} compared to native G_{D3} allowed its separation by HPLC on C_{18} reversed-phase columns. Utilizing isocratic elution with a linear water-acetonitrile gradient, cleaved G_{D3} was recovered first, and uncleaved G_{D3} was eluted in later fractions. The incubation of cleaved G_{D3} with proteins resulted in the formation of Schiff bases between the cleaved ganglioside and ε-aminolysyl groups. They were reduced with sodium cyanoborohydride to form stable secondary amine bonds (28). The reaction was monitored by HPTLC, which showed a decreasing ratio of the cleaved G_{D3} to a resorcinol positive band at the origin, indicating the formation of neoglycoconjugates. The reaction was generally completed after incubation for 48 h at 37°C. Disialyllactose was readily remov-

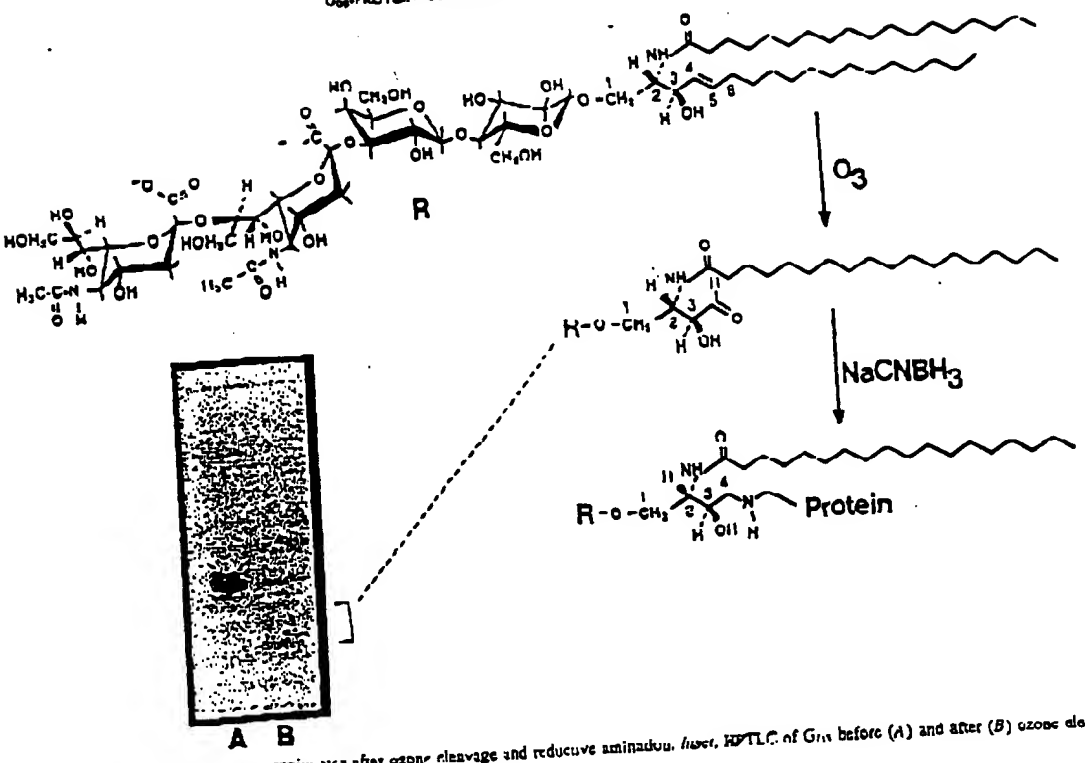


Fig. 1. Synthesis of G_{D3} protein conjugates after ozone cleavage and reductive amination. *Inset*, HPTLC of G_{D3} before (*A*) and after (*B*) ozone cleavage.

by dialysis, and the excess of cleaved G_{D3} was removed by ge through a detergent-removing column. The degree of cou- was determined by sialic acid and protein determinations. The ht ratio of G_{D3} to proteins in the different conjugates, shown in e 1, depended on the accessibility of lysine groups in the proteins. average yield of G_{D3} coupled to proteins was 30%. G_{D3} conju- s prepared in this way were reactive with anti-G_{D3} mAb R24 by tern blot analysis, although the G_{D3}-aldehyde derivative itself was reactive by ITLC (data not shown).

Oligosaccharide Conjugation. The carbohydrate part of G_{D3}, di- yllactose, was coupled to proteins utilizing two methods. The first hod, reductive amination, resulted in conjugation of the open ring n of the glucose to proteins (26). The method required a long ibation of the oligosaccharide with proteins, and the yield was less 120%. In the second method (27), involving *N*-acroloylation of the inal glucose, the oligosaccharide was coupled to proteins with a closed ring formation. None of these oligosaccharide conjugates showed reactivity with mAb R24 by Western blot analysis (data not shown).

Induction of a Serological Response against G_{D3} by Immunization with G_{D3}-Protein Conjugates. All vaccines were well tolerated. Mice were observed for at least 6 months, and neither acute nor systemic toxicity was detected. The serological response to immuni zation with G_{D3} or G_{D3}-protein conjugates, using QS-21 as adjuvant, is shown in Table 1. QS-21 was used because we had previously demonstrated its superiority over other adjuvants with another carbohydrate antigen-KLH conjugate vaccine (29). In ELISA, preimmuni zation sera showed no IgM or IgG antibodies reactive with G_{D3}. Immunization with unconjugated G_{D3} did not induce the production of G_{D3} antibodies. Immunization with G_{D3} conjugates, on the other hand, was effective in inducing antibody production. Of the five proteins used in the preparation of the conjugates, KLH showed the

Table 1 *Antibody response to immunization with different vaccines containing G_{D3} or disialyllactose conjugated to carrier proteins*

Vaccine + QS-21	No. of mice	G_{D3}:protein weight ratio[a]	Reciprocal ELISA peak titer against G_{D3}	
			IgG	IgM
G_{D3}	5	0.33	0 (5)	20 (3), 0 (2)
G_{D3}/KLH[b]	14	0.69	0 (5)	160, 40, 20 (3)
G_{D3}-KLH[c]	15	0.77	10,240 (2), 5,120 (2), 2,560 (3), 1,280 (7), 80 (2), 40 (2), 0	2,560, 1,280 (3), 640, 320 (3), 160 (2), 80 (3), 20, 0
G_{D3}-cBSA[c]	13	0.93	2,560, 1,280 (2), 640, 320 (4), 160, 80 (2), 40 (4), 20 (2), 0 (2)	80 (2), 40 (2), 20 (7), 0 (4)
G_{D3}-OMP[c]	10	1.0	40, 0 (9)	1,280, 320 (2), 160 (7), 80 (4), 40
G_{D3}-MAP[c]	10	ND	0 (10)	160 (2), 40 (4), 20 (3), 0
G_{D3}-Polylysine[c]	4	0.033	0 (4)	320, 160 (4), 80, 40, 20 (2), 0
Disialyllactose-KLH[d]	4	0.16	20, 0 (3)	160 (3), 80
Disialyllactose-cBSA[d]	4	0.25	20, 0 (3)	40, 20 (3)
Disialyllactose-KLH[e]	4	0.34	0 (4)	40 (2), 0 (2)
Disialyllactose-cBSA[e]	5	ND	0 (5)	0 (4)
Disialyllactose-Polylysine				80 (1), 40 (2)

[a] Protein and ganglioside content were determined by BioRad protein assay and by neuraminic acid determination according to the method of Svennerholm (25).
[b] G_{D3} and KLH were mixed prior to immunization.
[c] G_{D3} was covalently attached to proteins prior to immunization after ozonolysis as described in "Materials and Methods."
[d] Disialyllactose was conjugated to KLH and cBSA by reductive amination according to the method of Gray (26).
[e] Disialyllactose was conjugated to KLH, cBSA, and poly-L-lysine after *N*-acroloylation and Michael addition according to the method of Roy and Laferrière (27).

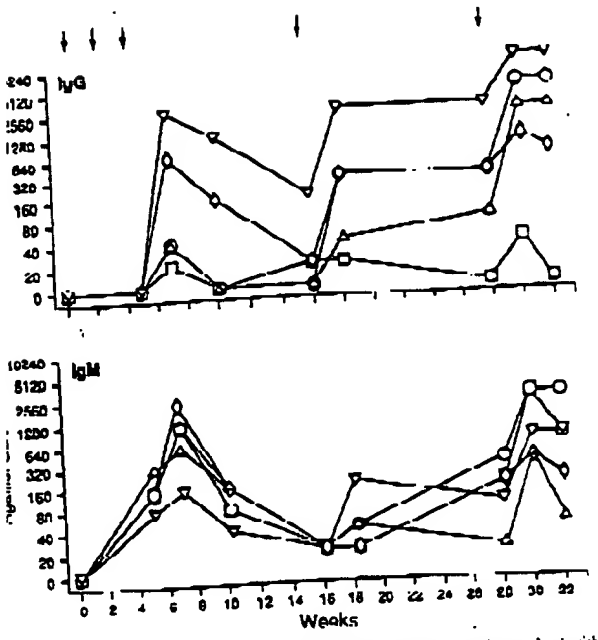


Fig. 2. Time course of G_{D3} antibodies induced in representative mice immunized with -KLH and QS-21 vaccine. Each symbol represents an individual mouse. Arrows, time accination.

ongest immunogenicity, resulting in a median titer of 1:320 for IgM d 1:2560 for IgG antibodies. The specific isotype profile was de- mined with subclass-specific secondary rabbit anti-mouse antibod- . Antigen-specific antibodies were found to be predominantly of the G1 subclass. Antigen-specific IgG2a and IgG2b antibodies were und only in traces, and no IgG3 or IgA antibodies were detected. In contrast to immunization with G_{D3} conjugates, immunization ith G_{D3}-oligosaccharide conjugates induced only a weak IgM re- ponse to G_{D3} and no IgG response.

Sequential IgM and IgG antibody titers against G_{D3} for five mice mmunized with G_{D3} KLH and QS-21 are shown in Fig. 2. IgM titers eaked 2 weeks after the third vaccination and declined by the time of ie first booster immunization at week 16. The first booster immuni ation had no significant impact on IgM titers, but the second booster mmunization at week 28 increased IgM titers to the peak level seen after the third vaccination of the initial series. IgG titers also rose up to 2 weeks after the third vaccination and decreased by the time of the first booster vaccination but rapidly increased after the booster to previous peak titers. IgG titers remained at this level for 10 weeks, with a further increase after the second booster in most mice. The evidence for a secondary immune response after the booster immunization was therefore equivocal. The response was clearly more rapid than after the initial immunization and lasted longer, but the increase in titer was not comparable to booster responses seen with classical T-cell-dependent antigens.

Specificity of the Serological Response to Immunization with G_{D3}-Protein Conjugates. The specificity of the serological response to immunization with G_{D3}-protein conjugates and QS-21 was analyzed by dot-blot immune staining and ITLC. An example of dot-blot immune stain analysis is shown in Fig. 3. Preimmune sera and immune sera showing high G_{D3}-antibody titers in ELISA were tested on nitrocellulose strips that had been spotted with G_{D3} (bbm) or G_{D3} (mel) and purified structurally related gangliosides: G_{M3}, G_{D2}, G_{D1b}, and G_{T3}. As expected on the basis of the ELISA results, preimmune sera showed no reactivity. In contrast, sera obtained after immunization with KLH conjugates of G_{D3}-ganglioside reacted with G_{D3} (bbm) (the immunogen) or G_{D3} (mel), but not with the other gangliosides except G_{T3} in some cases, a pattern also seen in tests of the mouse monoclonal IgG3 antibody R24, the reagent by which high cell surface expression of G_{D3} on human melanoma cells was first defined (20). The same specificity pattern was seen in dot-blot immune stain tests of sera from mice immunized with other G_{D3}-protein conjugates, the only exception being high-titer sera (by ELISA) from mice immunized with G_{D3}-cRSA, which showed no reactivity with G_{D3} or the other gangliosides.

ITLC permits specificity analysis of ganglioside antibodies in tests on tissue extracts. Examples of tests with high-titer sera from mice immunized with G_{D3}-KLH and QS-21 are shown in Fig. 4. The sera were tested at a dilution of 1:150 on ganglioside extracts of human brain, neuroblastoma, and melanoma, as well as G_{D3} (bbm) that had been used for immunization. The figure shows HPTLC ganglioside patterns of these reagents after staining with resorcinol, as compared with the patterns of reactivity exhibited after exposure to sera from immunized mice or mAb R24. As can be seen in the resorcinol-stained panel, the predominant gangliosides in the brain tissue extract are G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}, whereas the neuroblastoma extract shows G_{D2} and G_{M2} in addition, and the melanoma extract contains mainly

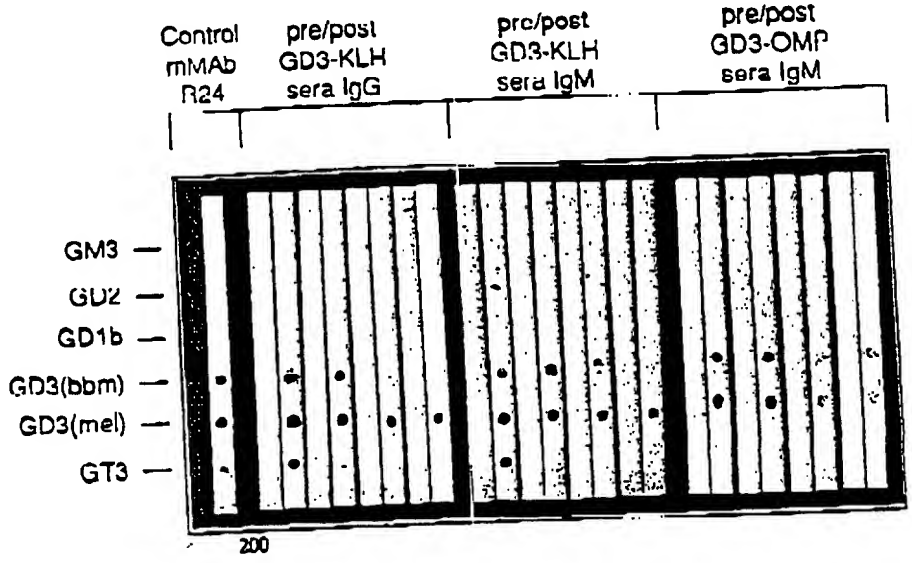
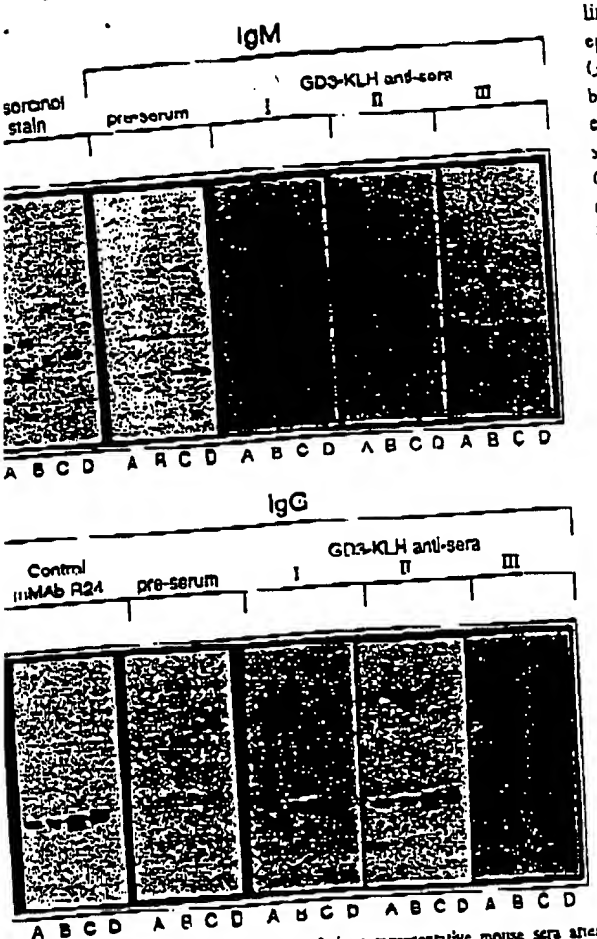


Fig. 3. Dot-blot immune stain assay for IgM and IgG antibodies in sera of mice immunized with G_{D3}-KLH and G_{D3}-OMP conjugates and QS-21. Antigen standards were applied to nitrocellulose strips in equal amounts (0.5 μg) and were allowed to react with pre/postimmunization serum from individual mice.



g. 4. Immune thin-layer chromatograms of three representative mouse sera after nation with G_{D3}-KLH conjugate and QS-21. IgG and IgM antibodies in pre- and ccination sera and anti-G_{D3} mMab R24 were tested on human brain gangliosides neuroblastoma gangliosides (*B*), melanoma gangliosides (*C*), and G_{D3} (*D*) (bbm). liosides were chemically stained with resorcinol-HCl reagent to demonstrate the ioside composition of each sample.

and G_{M3}. Reactivity of IgG antibodies in postimmunization sera, ell the reactivity of IgG3 mouse monoclonal antibody R24, was icted to G_{D3} (Fig. 4*b*). The high-titer IgM antibodies, on the other d, showed weak cross-reactivity with other gangliosides and sul- le in the brain extract (Fig. 4*a*).

era from mice immunized with other G_{D3} conjugates were tested ie same way (at lower dilution) and showed the same specificity i the exception, again, of high-titer sera from mice immunized i G_{D3}-cBSA, which showed no ganglioside reactivity (data not wn).

ell Surface Reactivity of Immune Sera Determined by FACS alysis. Sera from mice were tested for binding to cells of the anoma cell line SK-MEL-28, a cell line known to express cell ace G_{D3}. A representative example of a FACS analysis utilizing a rescein isothiocyanate-labeled secondary goat anti-mouse anti- y is shown in Fig. 5. Sera before and after immunization with -KLH and QS-21 were tested. Preimmunization serum stained 8% he target cells, postimmunization serum 92%.

ICUSSION

Conjugation of poorly immunogenic antigens to highly immunoic carrier molecules is a well-known approach to augmenting nunogenicity. Ganglioside molecules are so small, however, that linkage to carrier molecules without affecting the relevant antigenic epitopes is difficult. We have shown previously that modifications of (G_{D3} in its carbohydrate portion (i.e., conversion of sialic acid carboxyl groups to amides or gangliosidols or lactones) results in markedly increased immunogenicity. However, antibodies produced in response to these G_{D3} derivatives show no cross-reactivity with native G_{D3} (11, 30). Covalent attachment of proteins to the sialic acid molecules of G_{D3} was therefore not attempted in the present study. Our initial approach involved conjugation of G_{D3} oligosaccharide (disialyllactose) via the terminal glucose in open- or closed ring configuration to KLH or polylysine, but these conjugates were not recognized by the anti-G_{D3} mAb R24 or by mouse antisera to G_{D3}, and mice immunized with the conjugates did not produce G_{D3} antibodies. Subsequently, we coupled G_{D3} to proteins via its ceramide portion without alteration of the carbohydrate moiety. The ceramide was cleaved with ozone at the double bond of the sphingosin base, and coupling to proteins was accomplished by reductive amination. Cleavage of gangliosides by ozonolysis and subsequent conjugation with proteins by this method has not been described, and it has been generally assumed that the aldehyde intermediates of gangliosides would be unstable. Fragmentation, initiated by hydroxy ions under alkaline conditions, has been reported. Migration of the double bond would result in β-elimination, causing release of the oligosaccharide moiety (22, 31). We found, however, that the aldehyde was sufficiently stable at neutral pH to permit Schiff base formation with amino groups of proteins, so that β elimination was not a major problem. The overall yield was 30%. These G_{D3} aldehyde protein conjugates showed reactivity with G_{D3} antibodies by Western blot analysis, indicating that the immunodominant epitopes were intact in these G_{D3} conjugates. However, reactivity of the G_{D3}-aldehyde derivative with mAb R24 by ITLC could not be shown. This may be due to its relatively unstable nature, resulting in β-elimination and release of oligosaccharide during the immune stain incubation period, or simply to the fact that the G_{D3}-aldehyde derivative may not adhere to the thin-layer plate sufficiently for serological detection.

Earlier studies describe oxidative ozonolysis of the glycosphingolipid olefinic bond, resulting in a carboxyl group that could be conjugated with carbodiimide to NH_2 groups of modified glass beads, agarose gel, or other macromolecules (32, 33). This method, however, is of limited use for the conjugation of gangliosides to carrier proteins because it requires acetylated, methyl ester derivatives of gangliosides to avoid coupling via the sialic acid carboxyl group. Deacetylation after conjugation under basic conditions is necessary, conditions most proteins cannot be exposed to without degradation.

Once the conjugation method was established, several protein carriers were considered, based on previous work by others. Lowell *et al.* (34) described an elegant system that resulted in high-titer antibody responses as a consequence of anchoring bacterial carbohydrate and peptide antigens via a synthetic, hydrophobic foot in OMPs of *Neisseria meningitidis* (35). This system was directly applicable to gangliosides because of their amphipathic nature. In previous studies, we adsorbed gangliosides onto OMP by hydrophobic interaction, and we were able to induce high-titer IgM responses (36). Covalent attachment was utilized in the current study, but G_{D3}-OMP conjugates induced only occasional IgG responses, and the IgM response was not increased. Conjugation with cationized BSA, which has been reported to be a potent carrier for protein antigens (37), resulted in high-titer IgG antibodies detected by ELISA, but immune stains indicated that the response was not G_{D3}-specific. Another appealing carrier is the MAP system described by J. P. Tam (38, 39). MAPs consist of four or eight dendritic peptide arms, containing B- and T-cell epitopes, attached to an oligomeric branched lysine core. The antibody response to peptides was dramatically increased when these constructs were

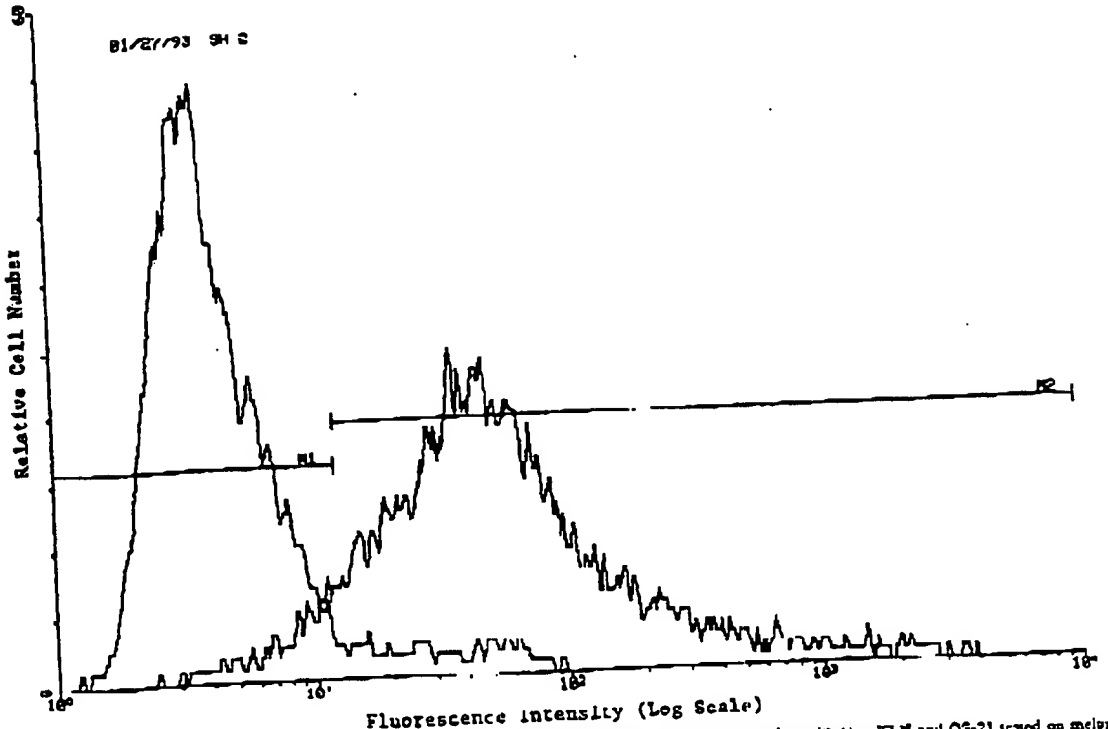


Fig. 5. Representative FACS analysis of mouse serum reactivity prior to (peak at 3) and after (peak at 50) immunization with G_{D3}-KLH and QS-21 tested on melanoma cell line SK-MEL-28.

used. When we attached G_{D3} to the amino terminal end of the MAP structure containing a malarial T-cell epitope, only a moderate IgM response against G_{D3} was detected, and there was no detectable IgG response. Conjugation of G_{D3} to polylysine resulted in a medium-titer IgM response and no IgG response, despite the high density of G_{D3} epitopes on these constructs.

The carrier that proved to be most effective in enhancing the antibody response to G_{D3} in this series was KLH. Immunization with G_{D3}-KLH consistently induced long-lasting production of IgM and IgG antibodies against G_{D3} at high titers. In comparing KLH with cBSA, OMP, MAP, and polylysine, it is difficult to know exactly why KLH is a superior carrier for G_{D3}. The sheer size and antigenic complexity of KLH stand out as a possible aid to antigen processing and recruitment of T-cell help across a broad range of T-cell specificities. The very qualities that make KLH cumbersome to work with are probably responsible for its unique effectiveness as a carrier in conjugate vaccines. KLH has not been widely used as a carrier for conjugate vaccines in humans because its size and heterogeneity make vaccine construction and standardization difficult.

Our hope was that conjugate vaccines would convert the T-cell-independent response against unconjugated G_{D3} seen in our previous studies to a T-cell-dependent response producing high-titer, long-lived, IgG antibodies. This expectation was fulfilled to some extent but not completely. The peak of the IgM response occurred after the third biweekly vaccination as in our previous studies with unconjugated G_{D3}, but the antibody titers were significantly higher. The response declined rapidly (as observed before), and additional vaccinations increased IgM titers to previous peak levels. The repeated increase in the titer of IgM antibodies to G_{D3} after booster immunizations differs from the expected response to T-cell-dependent antigens such as proteins, which generally induce little or no IgM response after booster immunizations. For the first time, however, we were able to induce a high-titer IgG response against G_{D3} ganglioside consistently. This response lasted significantly longer than the IgM response and was increased by additional vaccinations, although the response following booster vaccinations was not comparable to the exponential increase often seen with protein antigens. The fact that the G_{D3} antibodies were of the IgG1 subclass indicates that a T cell-dependent pathway was activated by the G_{D3}-KLH conjugate vaccine. The lack of a classical booster effect, however, may reflect the carbohydrate nature of G_{D3} and its status as an auto-antigen. This suggests that T cell recruitment by ganglioside conjugate vaccines is limited by the nature of the antigen itself. Nevertheless, the high-titer IgM response and long-lived IgG response to vaccination with G_{D3} KLH and QS-21 seen in these experiments represents a striking improvement over the response to unconjugated ganglioside vaccines and can now form the basis for clinical trials of ganglioside-KLH conjugate vaccines in patients with cancers that show increased ganglioside expression.

ACKNOWLEDGMENTS

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Exhibit D

KLH Conjugate Vaccine: Increased Immunogenicity in Melanoma Patients Administration with Immunological Adjuvant QS-21[1]

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ACT

cell surface gangliosides G_{M2}, G_{D2}, and G_{D3} are often overex- in malignant melanoma. We have shown previously that immu- n of melanoma patients with G_{M2} and *Bacillus Calmette-Guérin* d an IgM antibody response in most patients and that patients with ter G_{M2} antibodies showed increased survival. As is commonly seen arbohydrate antigens (which are T independent), the IgM response ort lived, and an IgG response was rarely observed. To increase nogenicity, we conjugated G_{M2} covalently with keyhole limpet he- anin (KLH). G_{M2}-KLH vaccine was given to melanoma patients or with one of the three adjuvants: *Bacillus Calmette-Guérin*, DE- , or QS-21. The most effective vaccine was G_{M2}-KLH with QS-21. It ed a much higher titer, a longer-lasting IgM G_{M2} antibody response. consistent IgG response (isotype IgG1 and IgG3). It also induced the st titer anti-KLH response. The results suggest that the conjugate -KLH plus QS-21 vaccine elicited significant T-cell help. Because e was no serious toxicity, this vaccine approach is attractive for nending the immunogenicity of other gangliosides, such as G_{D2} and , and to determine the effects of ganglioside antibodies on the course melanoma. In addition, the finding that QS-21 significantly increased immunogenicity of G_{M2}-KLH suggests that it may do the same for er conjugate vaccines, many of which are currently used without uvant.

TRODUCTION

One of the changes that occur in the process of malignant trans- mation is an altered pattern of cell surface ganglioside expression certain types of cancer, including malignant melanoma (1). In mal melanocytes, G_{M3}[3] is the predominant ganglioside. Other nglioslides, which include G_{D3}, G_{M2}, G_{D1a}, and G_{T1b}, constitute less n 10% of the total (2). In malignant melanoma, activation of cosylating enzymes leads to increased expression of G_{D3}, G_{D2}, , and 9-*O*-acetyl G_{D3} (3, 4). These overexpressed gangliosides are ractive targets for immunotherapy, including active immunization th ganglioside vaccines. In a series of studies involving G_{M2} vac- es in patients with malignant melanoma, we have shown that ccination (after low-dose cyclophosphamide and with BCG as juvant) induces IgM antibodies to G_{M2} in most patients (5), and that isease-free interval and survival are extended in patients producing high-titer G_{M2} antibodies (6, 7). However, the induced antibody response to G_{M2} has the characteristics of a T-independent response (predominantly IgM, short duration, inconsistent IgG response, and lack of booster effect), and the other melanoma gangliosides, G_{D3} and G_{D2}, are not immunogenic when administered in the same way (8). As the relevant epitopes are carbohydrates, we have explored approaches to increasing immunogenicity that are suggested by the successful development of carbohydrate vaccines for bacterial infections. In the mouse, we have shown that the immunogenicity of G_{D3} is markedly increased by covalent binding to KLH and that mice immunized with the G_{D3}-KLH conjugate and the adjuvant QS-21 show a high-titer IgM response, followed by a strong, long-lasting IgG response (9). We have now begun to test ganglioside conjugate vaccines in mela- noma patients and report here the results of initial studies with vaccines containing G_{M2}-KLH conjugate plus various adjuvants.

MATERIALS AND METHODS

Patients

Forty-eight patients with malignant melanoma stage III or stage IV who were free of detectable disease as a consequence of surgery within the previous 4 months were treated. None of the patients had received prior chemotherapy or radiation therapy. Six of the 30 patients receiving G_{M2}-KLH plus QS-21 were part of an initial Phase I study and have been described previously (10).

Vaccine Preparation and Administration

G_{M2}-KLH Vaccine. G_{M2}-KLH conjugate was prepared by Biomira, Inc. and in our laboratory as described previously for G_{D3}-KLH conjugate vaccine (9). Briefly, the conjugation procedure involved ozone cleavage of the ceram- ide double bond of G_{M2}, introduction of an aldehyde group, and conjugation to aminolysyl groups of KLH by reductive amination. The G_{M2}:KLH molar ratio was approximately 800:1, and one patient dose contained 70 μg G_{M2} and approximately 500 μg KLH in 0.5 ml of normal saline. Groups of six patients each received G_{M2}-KLH conjugate without adjuvant, G_{M2}-KLH with DETOX, and G_{M2}-KLH with BCG, and thirty patients received G_{M2}-KLH with QS-21.

Four vaccinations were administered intradermally into extremities with intact lymphatic drainage at 2-week intervals, followed by two additional vaccinations at 8-week intervals. Cyclophosphamide (Cytoxan; Mead Johnson and Co., Evansville, IN; 200 mg/m²) was administered i.v. to all patients 4 to 6 days before the first vaccination.

Immunological Adjuvants. DETOX was produced and supplied by Ribi Immunochem Research, Inc. (Hamilton, MT) formulated as a lyophilized oil droplet emulsion. It consists of CWS from BCG and MPLA from *Salmonella minnesota* R595. On the day of vaccination, 0.25 ml DETOX (250 μg CWS + 25 μg MPLA) was mixed with the G_{M2}-KLH preparation. The vaccine (final volume, 0.75 ml) was vortexed for 2–3 min and administered to the patients within 15 min. BCG was purchased from Bionetics Research, Inc. (Rockville, MD). On the day of vaccination, 10^7 viable units of BCG in 0.1 ml normal saline were added to the G_{M2}-KLH vaccine in each individual syringe (final volume, 0.6 ml). The contents were mixed and administered to the patients within 15 min. QS-21 adjuvant (a homogeneous saponin purified from the bark of *Quillaja saponaria* Molina; Refs. 11 and 12) was provided by Cambridge Biotech, Inc. (Worcester, MA). QS-21 (100 or 200 μg) were diluted in 0.25 ml normal saline and mixed with G_{M2}-KLH. The vaccine (final volume, 0.75 ml) was vortexed for 2–3 min and administered within 15 min.

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To whom requests for reprints should be addressed, at Active Biotherapies Inc. a ubsidiary of Progenics Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, Y 10591.
The abbreviations used, in accordance with the ganglioside nomenclature proposed Svennerholm (13), are: G_{M3}, II^3NeuAc-GalCer; G_{M2}, II^3NeuAc-GgOse$_2$Cer; G_{M1}, ^3NeuAc-GgOse$_4$Cer; G_{D3}, $II^3(NeuAc)_2$-GgOse$_2$Cer; G_{D2}, $II^3(NeuAc)_2$-LacCer; G_{D1a}, $^3NeuAc,II^3NeuAc$-GgOse$_4$Cer; G_{D1b}, $II^3(NeuAc)_2$-GgOse$_4$Cer; and G_{T1b}, $II^3(NeuAc)_2$, $^3(NeuAc)GgOse_4Cer$. Other abbreviations used are: BCG, *Bacillus Calmette-Guérin*; KLH, whole limpet hemocyanin; CWS, cell wall skeletons; MPLA, monophosphoryl lipid A; Hib, *nemophilus influenzae* type b; PRP, phosphoribosyl/ribulolphosphate polysaccharide; PTLC, high performance thin layer chromatography.

gangliosides

G_{M2} from bovine brain was received from Fidis Research Laboratory ...sano Terme, Italy) or was isolated from Tay-Sachs cat brains in our ...oratory by published procedures. G_{M3}, G_{M1}, G_{D1a}, and G_{T1b} from bovine ...in were purchased from Sigma Chemical Co. (St. Louis, MO). Asialo-G_{M2} ...is prepared by treatment of G_{M2} with 0.1 M trifluoroacetic acid at 100°C for ...h, followed by separation on a reversed phase column (Sep-Pak C_{18}; Waters, ...ilford, MA). G_{D2} was made from G_{D1a} by treatment with β-galactosidase, ...as was isolated from bovine buttermilk and kindly provided by Dr. R. K. Yu ...Medical College of Virginia, Richmond, VA).

Reagents and Monoclonal Antibodies

HPTLC silica gel plates were obtained from E. Merck (Darmstadt, Germany); 4-chloro-1-naphthol and p-nitrophenyl phosphate disodium were obtained from Sigma. Alkaline phosphatase-conjugated goat anti-human IgM (Kirkegaard and Perry Labs, Gaithersburg, MD) and mouse anti-human IgG (Southern Biotech, Birmingham, AL), followed by alkaline phosphatase-conjugated goat anti-mouse IgG (Southern Biotech), were used for ELISA. Horseradish peroxidase-conjugated goat anti-human IgM or IgG purchased from TAGO (Burlingame, CA) was used for dot blot immune stain and immune thin layer chromatography. Rabbit anti-mouse immunoglobulins conjugated to horseradish peroxidase for immune thin layer chromatography and rabbit anti-mouse IgM and IgG conjugated to alkaline phosphatase for ELISA were used with control monoclonal mouse antibodies and were obtained from Zymed (San Francisco, CA). Murine anti-G_{M2} mAb 696 (IgM) was kindly provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan; Refs. 13 and 14), and anti-G_{D2} mAb R24 (IgG3) was generated in our laboratory (15).

Serological Assays

ELISA was performed as described previously (6). To control for nonspecific "stickiness," immune sera were also tested on plates that were processed identically but to which no ganglioside had been added, and the reading was subtracted from the value obtained in the presence of ganglioside. The titer was defined as the highest dilution yielding a corrected absorbance of 0.1 or greater. Immunostaining of gangliosides with mAbs or human sera was performed after spotting on nitrocellulose strips (16) or separation on HPTLC silica gel glass plates as described previously (3). Plates were developed in chloroform:methanol:water (0.25% $CaCl_2$) 50:40:10 (v/v), and gangliosides were visualized by staining with resorcinol/HCl reagent or mAbs.

Determination of IgG Subclass

Determination of IgG subclass was performed by ELISA using subclass-specific secondary mouse anti-human IgG1, IgG2, IgG3, and IgG4 mAbs. Secondary mAbs from different suppliers (Table 2) were used. Alkaline phosphatase conjugated to goat anti-mouse IgG (Southern Biotech) was used as third antibody at a dilution of 1:200.

Complement-mediated Cytotoxicity Assays

Complement-mediated cytotoxicity assays were performed by a 4 h ^{51}Cr release assay. Cells from the G_{M2}-positive melanoma cell line SK-MEL-173 served as target cells. Cells (2×10^6) were labeled with 100 μCi $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA) in 10% FCS RPMI for 1 h at 37°C in a CO_2 incubator. The cells were washed twice, and 10^4 cells/well in 96-well round-bottomed plates (Corning, New York, NY) were labeled and incubated with 1:5 diluted pre- or postvaccination serum or with medium alone for 1 h at 37°C in a CO_2 incubator. The cells were washed and incubated with human complement (Sigma) at a dilution of 1:4 for 4 h at 37°C. The plates were spun at 500 × g for 5 min, and an aliquot of 125 μl of supernatant of each well was harvested for determination of released ^{51}Cr. All assays were performed in triplicate and included control wells for maximum release in 1% NP40 (Sigma) and for spontaneous release in the absence of complement. The percentage of specific lysis was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100$$

Statistical Analysis

Titers between groups were compared using the Wilcoxon rank-sum test. Because the purpose of the study was to generate rather than to test hypotheses, the Ps were not adjusted for multiple comparisons.

RESULTS

Vaccine Administration and Side Effects. Forty-eight patients were immunized with the G_{M2}-KLH vaccine. Groups of 6 patients each received G_{M2}-KLH with no immunological adjuvant or with DETOX and BCG, and 30 patients received G_{M2}-KLH with QS-21. No local or systemic toxicity was detected after administration of G_{M2}-KLH alone. Vaccines containing DETOX resulted in nodule formation at vaccination sites in four of six patients that lasted 2–10 weeks. In four patients, these were associated with 3–10 cm of erythema and induration but only minimal tenderness. In one patient, it was associated with 25 cm erythema and induration after one immunization, and in a second patient, low grade fever and malaise for 72 h after the first immunization. In this patient, the DETOX dose was reduced to 50 μg CWS + 5 μg MPLA for the subsequent immunizations. BCG produced local inflammation and crusting at some point in all patients, which healed after 2–12 weeks. When this occurred, the dose of BCG was reduced from 1×10^7 viable units to a final dose of 3×10^6 units in four patients and 1×10^6 units in one patient. The sixth patient had a history of tuberculosis exposure and a positive purified protein derivative (PPD) test and was, therefore, started at a dose of 1×10^6 units, which was eventually reduced to 1×10^5 units. QS-21 induced mild local erythema, induration, and tenderness lasting 24–72 h in all patients at the 100-μg dose. The 200-μg dose of QS-21 was associated with local tenderness and inflammation lasting 2–10 days in all patients as well as mild flu-like symptoms, including low grade fever (<38.5°C), headache, and myalgia lasting 8–24 h after most immunizations. No neurological abnormalities or other side effects were observed.

Antibody Response to G_{M2}-KLH Conjugate Vaccines. Before vaccination, IgG antibodies against G_{M2} were not detected, and IgM antibodies were detected only rarely. IgM titers of 1:40 were seen in three patients, and two patients had a pretreatment titer of 1:320. The remaining 43 patients showed G_{M2} reactivity with 1:20 titers or lower before vaccination. ELISA and immune stain results with sera obtained before and after immunization are summarized in Table 1. The IgM antibody titers after immunization with G_{M2}-KLH or with G_{M2}-KLH and DETOX or BCG were quite similar (median titer, 1:80–1:240; $P > 0.15$ between any pair of groups). In contrast, 25 of 30 patients immunized with G_{M2}-KLH and QS-21 showed IgM antibody titers of 1:320 or more, significantly higher than the titers in the other groups ($P < 001$, $P = 0.02$, and $P = 0.06$) or in patients immunized with previous G_{M2}/BCG vaccines ($P < 0.001$; Ref. 6). In addition, immunization with G_{M2}-KLH and QS-21 induced a consistent IgG response for the first time; only 5 of the other 18 patients receiving G_{M2}-KLH vaccines produced comparable IgG titers.

Median sequential IgM and IgG antibody titers against G_{M2} in patients receiving G_{M2}-KLH alone or with adjuvants DETOX and BCG and the first six patients of the G_{M2}-KLH plus QS-21 group are shown in Fig. 1. IgM peak titers were seen after the third or fourth vaccination and remained elevated in most patients receiving the QS-21 vaccine for at least 20 weeks. Booster immunizations at weeks 14 and 22 did not further increase IgM titers. IgG titers of 1:160 or higher were seen 2 weeks after the fourth vaccination in five of six patients receiving the QS-21 vaccine. The titers decreased to 1:40 or less but rapidly increased again after booster vaccination to the previous levels (median 1:160) and remained at this level for more

Table 1 *Serological response of patients receiving G_{M2}-KLH conjugate vaccines with or without adjuvants in comparison to vaccine [illegible] G_{M2} adherent to BCG (G_{M2}/BCG)*

Vaccine	No. of patients	Reciprocal G_{M2} antibody titers: Before immunization IgM	Before immunization IgG	After immunization (peak) IgM	After immunization (peak) IgG	Dot blot immune stain for G_{M2} antibodies IgM	Dot blot immune stain for G_{M2} antibodies IgG	Reciprocal KLH antibody titers after immunization (peak) IgG
KLH	6	10(2),0(4)	0(6)	320,160,80(2),40,20	10,0(5)	3+(2),2+(2),0(2)	1+(2),0(4)	360,180(3),20(2)
dian titers		0	0	80	0			180
KLH + DETOX	6	20(2),10(2),0(2)	0(6)	640(2),160(3),40	160,0(5)	3+(2),2+(3),1+	2+,0(5)	1080(2),360,180(3)
dian titers		10	0	160	0			270
KLH + BCG	6	40,20,0(4)	0(6)	1280,320(2),160(2),40	320,20(2),0(3)	3+,2+(3),0(2)	1+(2),0(4)	3240,1080(2),360(2),180
dian titers		0	0	240	10			720
KLH + QS-21	30	320(2),40(7)	0(30)	>1440(2),1280(13),640(4),	1280(2),640(5),320(5)	3+(28),2+(2)	3+(16),2+(5)	7290(4),2430,810
		20(11),0(15)		320(6),160(3),80(2)	160(8),80(2),40(5),20,10(2)		1+(3),0(6)	
dian titers		10	0	960	160			7290
/BCG[b]	58	160,40,20(10),	0(58)	640(11),320(9),	640,160,80(4),	3+(32),2+(15),	3+(2),2+(5),	ND
		10(11),0(35)		160(15),80(8),40(8)	20,10(3),0(48)	1+(4),0(7)	1+(4),0(47)	
dian titers		0	0	20(2),10(3),0(2)	0			ND
				160				

Only the first six patients were analyzed for anti-KLH antibodies. ND, not done.
Historical data (6).

n 11 weeks. The second booster vaccination had no clear effect on ibody titers in most cases. Thus, the response to booster vaccina- n showed only one of two characteristics of the classical secondary mune response. The response occurred more rapidly, but antibody ers did not rise higher then after the initial immunization.

KLH antibodies were not detected in pretreatment sera. After accination, all patient sera showed reactivity with KLH as indicated , Table 1. The highest titers of IgG antibodies were seen after dministration with QS-21 (the first six patients were tested), signif- cantly higher than in all other groups, including the next-best group f patients vaccinated with G_{M2}-KLH and BCG ($P = 0.006$). In the QS-21 group, there was no correlation between the strength of the G_{M2} response and the KLH response.

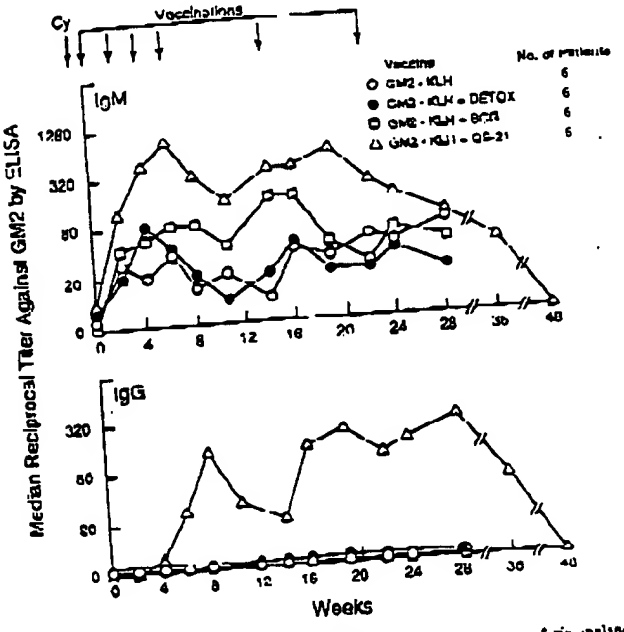

Fig. 1. Median IgM and IgG antibody titers over time in groups of six melanoma patients after immunization with G_{M2}-KLH alone or G_{M2}-KLH plus immunological adjuvants DETOX, BCG, and QS-21. Arrows, time of vaccine injections.

Specificity Analysis of G_{M2} Antibodies. The specificity of ganglioside antibodies detected in the patients' sera before and after immunization was determined by dot blot immune stains using the ganglioside standards G_{M3}, asialo-G_{M2}, G_{M2}, G_{M1}, G_{D2}, G_{D3}, G_{D1a}, and G_{D1b} (Fig. 2; first six patients of G_{M2}-KLH plus QS-21 group are shown). Preimmunization IgM and IgG antibodies from most patients showed weak reactivity with asialo-G_{M2}, and some patients also had IgM antibodies against G_{M1} and G_{D1b}. Reactivity with these gangliosides was not altered by immunization. The only vaccine-induced changes were strong reactivity with G_{M2} and weak reactivity with G_{D2}. Dot blot immune stains were graded as 0, 1+, 2+, or 3+. Reactivity of 3+ for IgM antibodies against G_{M2} was seen in the serum of 28 of 30 patients immunized with G_{M2}-KLH and QS-21, in 1 of 6 patients treated with G_{M2}-KLH and BCG, and in 2 of 6 patients treated with G_{M2}-KLH without adjuvant or G_{M2}-KLH and DETOX. Reactivity of 3+ for IgG antibodies was seen in 16 of 30 patients immunized with G_{M2}-KLH and QS-21 and in none of the patients in the other treatment groups.

Postvaccination sera from the first six patients immunized with G_{M2}-KLH and QS-21 were also tested by immune thin layer chromatography (Fig. 3) for reactivity with G_{M2} and other gangliosides of a melanoma tissue extract. Most patients' sera showed strong IgG and IgM reactivity with G_{M2} isolated from bovine brain or melanoma. Antisera reactivity was seen also with a lower migrating band in melanoma extract, presumably G_{D2}.

To confirm the G_{D2} cross-reactivity of IgG antibodies, postvaccination serum from patient no. 2 was preincubated with either G_{M2} or G_{D2} before performing the immune stain (Fig. 4). Reactivity with G_{M2}, and with G_{D2} in the melanoma ganglioside extract, was completely inhibited by preincubation with G_{M2}. On the other hand, preincubation of the same serum with G_{D2} resulted in inhibition of G_{D2} reactivity only and did not change reactivity with G_{M2}. These results suggest the presence of two populations of antibodies, one reacting with G_{M2} alone and another with reactivity for G_{M2} and G_{D2}.

Subclass Determination of IgG Antibodies. IgG sera from the first six patients immunized with G_{M2}-KLH and QS-21 were tested by ELISA using a panel of IgG subclass-specific secondary antibodies. The results are summarized in Table 2. The IgG antibodies in all six sera tested were of IgG1 and IgG3 subclass.

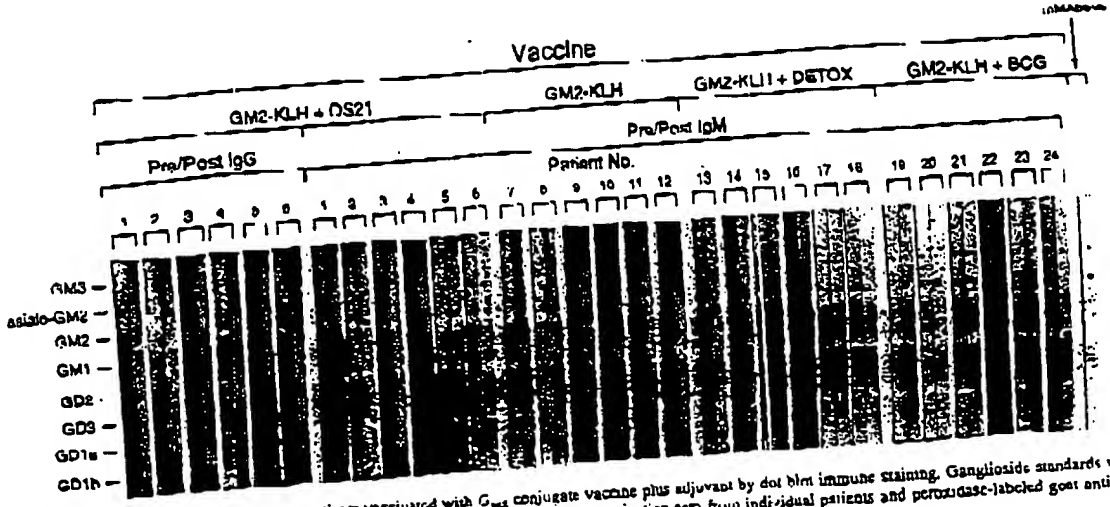

Fig. 2. Detection of G_{M2} antibody in sera from patients vaccinated with G_{M2} conjugate vaccine plus adjuvant by dot blot immune staining. Gangliosides standards were spotted on ...trocellulose strips (*ordinate*) and allowed to react with prevaccination and peak titer postvaccination sera from individual patients and peroxidase-labeled goat anti-human IgM or ...G antibody. Strips are graded on a scale from 0 to 3+. mAb 696 was used as positive control for G_{M2}.

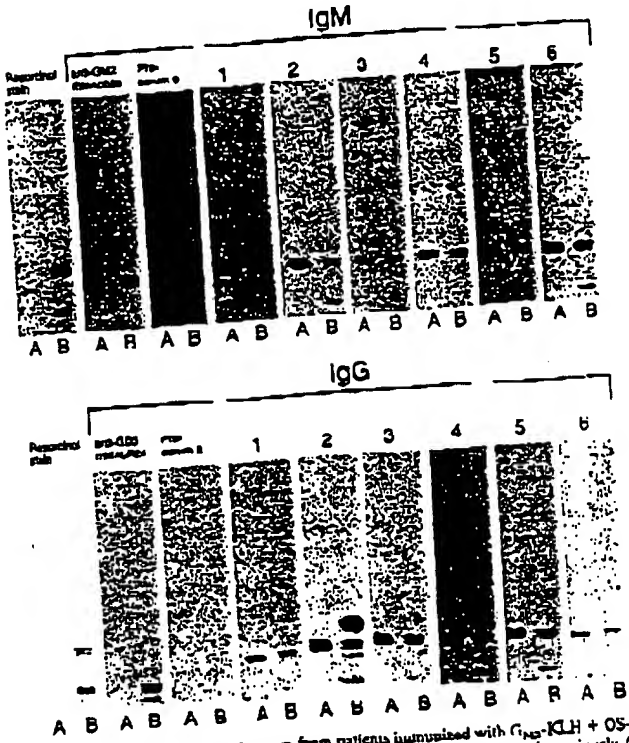

Fig. 3. Specificity of peak titer sera from patients immunized with G_{M2}-KLH + QS-21 vaccine determined by immune thin layer chromatography as described previously (3). G_{M2} (*A*) and melanoma tissue ganglioside extract (*B*) were applied to HPTLC plates, incubated with sera from individual patients, and stained with peroxidase-labeled goat anti-human IgM or IgG antibody. mAb 696 was used as positive control for G_{M2} and resorcinol stain for gangliosides.

Complement-mediated Cytotoxicity. Effector function of anti-G_{M2} antibodies in the serum of the first six patients vaccinated with G_{M2}-KLH and QS-21 (diluted 1:5) was tested by complement-mediated cytotoxicity assays. As shown in Table 3, postvaccination sera of all six patients lysed G_{M2}-positive SK-MEL-173 melanoma cells in the presence of human complement. Prevaccination sera showed no cytotoxicity with complement, and postvaccination sera were not cytotoxic when complement was not added. More detailed study of cell surface binding and cytotoxic effector functions of vaccine-induced antibodies and their subclasses is under way.

DISCUSSION

In a series of studies in patients with malignant melanoma, one objective has been to construct vaccines that are effective in inducing production of antibodies against three gangliosides often overexpressed in melanoma: G_{M2}, G_{D2}, and G_{D3}. Our initial approach was to vaccinate patients with unconjugated gangliosides adsorbed to BCG. In this way, we were able to induce antibody production against G_{M2} (5, 6) but not G_{D2} or G_{D3}. G_{M2} antibodies induced by G_{M2}/BCG vaccines were mostly of the IgM class, the antibody response was of short duration, and booster immunization resulted again in a brief period of IgM antibody production similar to the primary response; all characteristics of a T-cell-independent immune response, well known from studies of other carbohydrate antigens. Even so, vaccine-induced production of G_{M2} antibodies by patients with stage III melanoma after surgery was associated with increased survival (6, 7). This

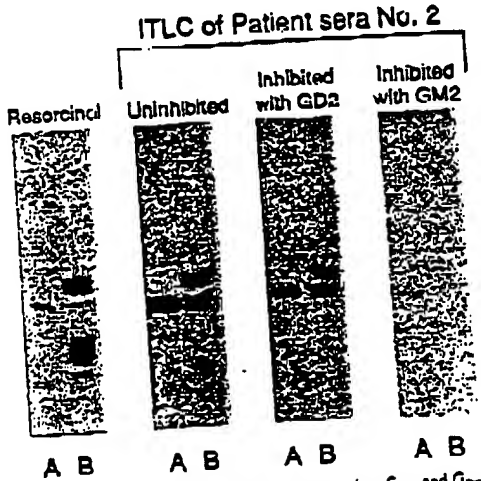

Fig. 4. Inhibition of IgG reactivity of patient serum against G_{M2} and G_{D2}. G_{M2} (*A*) and melanoma tissue ganglioside extract (*B*) were applied to HPTLC plates, incubated with serum from patient no. 2, and stained with peroxidase-labeled goat anti-human IgG antibody. Patient serum (3 ml) at a dilution of 1:50 was preincubated (inhibited) with either 150 μg G_{M2} or 150 μg G_{D2} prior to immune staining.

n suggested that melanoma gangliosides are appropriate ; for vaccine construction and that melanoma ganglioside of increased immunogenicity might result in superior clinical . Because the relevant epitopes of melanoma gangliosides hydrates, it is helpful to consider what efforts have been attempts to increase the immunogenicity of carbohydrate , notably against certain bacterial infections.

ajor distinction of the immune response to carbohydrate , as opposed to protein antigens, is that it does not depend on ius. The concept that carbohydrate antigens are thymus inde- is based on the observation that neonatally thymectomized well as athymic mice show unimpaired humoral immune es to bacterial polysaccharides (17). B cells that respond to -independent antigens show several characteristic features. ppear later in ontogeny, are long-lived, and do not require T r activation, at least not *in vivo*. Although T cells are required cells to respond to thymus-independent antigens *in vitro*, the of the T-cell effect is poorly understood and clearly different the MHC-restricted T-cell help in the T dependent antibody se to protein antigens. Although T-cells are not indispensable *in vivo* antibody response to thymus-independent antigens, anti- levels are higher when T cells are present, suggesting a general enting activity of T cells, again by unknown mechanisms (18).

large variety of approaches has been explored in attempts to ase the immunogenicity of carbohydrate antigens. They include ical modification (19), administration with adjuvants, noncova- complexing with proteins, covalent attachment to immunogenic in carriers (20), and replacement of the carbohydrate epitope by otein replica, either peptides synthesized *de novo* (so-called mim- es; Ref. 21) or antiidiotypic antibodies (22). Most of these ap ches result in increased T-cell help for the carbohydrate-specific body response. While each has shown promise in initial experi itation, covalent attachment of carbohydrate antigens to immuno- ic T-dependent protein carriers, as first suggested for haptens (23) l then disaccharides (24), is the concept that has been pursued most orously, resulting in vaccines that have in some instances been own to be highly effective in recent clinical trials.

Excellent examples are Hib polysaccharide protein conjugate vac ies. Four vaccines that have been developed over the last decade ffer in the carbohydrate compounds, the protein carriers, and the kers between carbohydrate and protein (25–29). In comparative

Table 2 *Characterization of IgG antibodies induced against G_{M2} with G_{M2}-KLH plus QS-21 vaccine by IgG subclass-specific mAbs*

IgG subclass mAbs			Reciprocal ELISA titer against G_{M2}					
			Patient no.					
Specificity	Conc.[a] (μg/ml)	mAbs source[b]	1	2	3	4	5	6
IgG	10	SBA[c]	640	640	640	640	640	640
	20	BS	40	20	20	n.d.	20	20
	2	ZLI	640	640	640	640	640	640
IgG1	10	SBA	10	10	10	10	10	10
	20	BS	10	10	0	10	0	0
	2	ZLI	10	0	10	0	10	0
IgG2	10	SBA	10	0	0	0	0	0
	20	BS	0	0	0	0	0	0
	2	ZLI	0	0	0	0	0	0
IgG3	10	SBA	20	20	20	20	20	20
	20	BS	40	80	40	40	40	40
	2	ZLI	20	40	20	10	10	20
IgG4	10	SBA	0	0	0	0	0	0
	20	BS	0	0	0	0	0	0
	2	ZLI	0	0	0	0	0	0

[a] Conc., concentration.
[b] SBA, Southern Biotechnology Associates (Birmingham, AL); BS, The Binding Site, Ltd.; ZLI, Zymed Laboratories, Inc. (San Francisco, CA).

Table 3 *Complement lysis of melanoma cell line 3A [illegible] antibodies in sera from patients immunized with G_{M2} KLH plus QS-21*

Patient no.	Percentage of lysis[a]		
	Prevaccination serum with complement	Postvaccination serum with complement	Postvac. serum without complement
1	1.3	38.75	3.1
2	2.2	16.9	2.7
3	1.1	14.0	0.9
4	1.2	26.0	2.2
5	2.1	34.9	1.7
6	10.5	64.7	2.3

[a] Target cells were labeled with ^{51}Cr and treated with 1:5 diluted antisera. Vac., vaccination.

studies in children, all conjugate vaccines induced a much stronger antibody response than unconjugated Hib PRP vaccine (30). Of particular interest are observations that young children first immunized with HbOC (oligosaccharide-nontoxic diphtheria toxin) or PRP-OMPC (outer membrane protein complex of *Neisseria meningitidis* type B) vaccines and later challenged with unconjugated PRP vaccine showed an anamnestic IgG response, even if challenged at an age at which they do not respond to primary immunization with the unconjugated vaccine (31, 32). How T cells are engaged and how they interact with Hib PRP-responsive B-cells is still far from clear. The fact that increased immunogenicity and T dependence require a covalent bond between PRP and protein suggests that the proximity between protein and PRP must not be disturbed, at least not in the early phase of antigen processing. As the isotype and biological activities of antibodies induced by Hib PRP and Hib PRP conjugates are the same, it appears that the B cells that respond to the conjugate-induced T-cell signal are qualitatively identical with those engaged by Hib PRP alone. Drawing on the substantial experience that has accumulated in the development of carbohydrate vaccines for bacterial infections, we have explored, over the past 5 years, similar approaches in our attempts to increase the immunogenicity of melanoma gangliosides. Chemical modification of G_{D3}, resulting in lactone, amide, or gangliosidol formation, produced derivatives that were highly effective in inducing antibody production. However, the antibodies induced by G_{D3} lactone, G_{D3} amide, or G_{D3} gangliosidol did not cross-react with G_{D3} (33, 34). An antiidiotypic antibody BEC-2, mimicking G_{D3}, was developed by immunizing mice with the monoclonal antibody R24, which recognizes G_{D3}. Rabbits immunized with BEC-2 produced anti-G_{D3} antibodies (35), and initial studies of the immunogenicity of BEC-2 in human patients are under way.

Regarding conjugate vaccines, our initial studies with G_{D3} in the mouse were concerned with three issues: development of the conjugation method; selection of the carrier protein; and choice of the adjuvant (9). The optimal conjugation procedure involved ozone cleavage of the double bond of G_{D3} in the ceramide backbone, introduction of an aldehyde group, and coupling to protein aminolysyl groups by reductive amination. Of five carriers tested, poly-L-lysine, KLH, cationized BSA, *Neisseria meningitidis* outer membrane protein complex, and multiple antigenic peptide containing four repeats of a malarial T-cell epitope, KLH was found to be most effective. Nonconvalent G_{D3}/KLH complexes were not immunogenic. The best adjuvant was QS-21, a homogeneous saponin fraction purified from the bark of *Quillaja saponaria* Molina. The characteristics of the antibody response to immunization with G_{D3}-KLH conjugate and QS-21 included: (*a*) a high initial antibody titer; (*b*) a rapid secondary rise of IgM antibody titers after booster immunizations; (*c*) maintenance of IgM antibody titers after booster immunizations for up to 10 weeks; and (*d*) consistent production of IgG antibody at high titers, parallel to IgM antibody production, except for the initial delay of 2 weeks. These findings have now been reproduced in human melanoma pa-

s by immunization with another ganglioside conjugate vaccine, -KLH, using the same conjugation procedure. As in the mouse lies, QS-21 proved to be a significantly more effective adjuvant DETOX or BCG, with acceptable toxicity.

he G_{M2} antibody response had many characteristics of a T-cell-ndent response. It was long-lasting, and antibodies of IgG1 and 3 subclass (usually associated with a T-cell-dependent immune ponse) were induced. As seen with the Hib-PRP vaccines, these types were the same as those induced occasionally at low titers th unconjugated G_{M2}-BCG vaccines. The lack of a clear booster fect in the sustained high-titer IgM and IgG response after vacci- tions 3 and 5 months following the initial series may be explained the fact that the patients were immunized at 2-week intervals ually. In the classical experiment showing the secondary response protein antigens, the second injection of antigen is given 4 weeks ter the first. Antibody levels after the first immunization are higher etween 1 and 2 weeks after the injection and then decline to very low evels before the booster injection is given after 4 weeks. In the mmunization schedule we chose, the initial antibody response did not ubside but increased in a stepwise fashion in response to the first four vaccinations at the 2-week intervals, anticipating the secondary re- sponse that is seen in a more dramatic fashion in the classical exper- ment. Unlike the antibody response to most protein antigens, the IgM response was long-lasting, and IgM antibodies remained at higher titer than IgG antibodies, even after repeated booster immunizations, as is characteristic for carbohydrate antigens. Hence, the immune response against gangliosides that contain a comparably short oligosaccharide chain linked to a lipid backbone and that are autoantigens show much in common with the immune response against Hib-PRP and other bacterial carbohydrates.

The development of the G_{M2}-conjugate vaccine will make it possible to determine whether higher levels of IgM and IgG antibodies against G_{M2}, sustained over longer periods, will be more effective in delaying recurrence of melanoma than the lower levels of mostly IgM antibodies present for shorter periods, in patients immunized with unconjugated G_{M2}. In addition, we can now test whether conjugation with immunogenic protein carriers also confers immunogenicity to G_{D3} and G_{D2}, major gangliosides which have not induced a consistent antibody response in melanoma patients when given as unconjugated vaccines. If this can be accomplished, construction and testing of a polyvalent melanoma ganglioside vaccine would be an attractive next step.

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Exhibit E

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Induction of Antibodies against GM2 Ganglioside by Immunizing Melanoma Patients Using GM2-Keyhole Limpet Hemocyanin + QS21 Vaccine: A Dose-Response Study[1]

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ABSTRACT

In a previous randomized Phase III trial (P. O. Livingston *et al.*, J. Clin. Oncol., *12*: 1036–1044, 1994), we demonstrated that immunization with GM2 and *bacille Calmette-Guérin* reduced the risk of relapse in stage III melanoma patients who were free of disease after surgical resection and who had no preexisting anti-GM2 antibodies. That vaccine formulation induced IgM anti-GM2 antibodies in 74% but induced IgG anti-GM2 antibodies in only 10% of the patients. To optimize the immune response against GM2, a reformulated vaccine was produced conjugating GM2 to keyhole limpet hemocyanin (KLH) and using the adjuvant QS21 (GM2-KLH/QS21). In pilot studies, 70 μg of vaccine induced IgG anti-GM2 antibodies in 76% of the patients. We wished to define the lowest vaccine dose that induced consistent, high-titer IgM and IgG antibodies against GM2. Fifty-two melanoma patients who were free of disease after resection but at high risk for relapse were immunized with GM2-KLH/QS21 vaccine at GM2 doses of 1, 3, 10, 30, or 70 μg on weeks 1, 2, 3, 4, 12, 24, and 36. Serum collected at frequent and defined intervals was tested for anti-GM2 antibodies. Overall, 88% of the patients developed IgM anti-GM2 antibodies; 71% also developed IgG anti-GM2 antibodies. GM2-KLH doses of 3–70 μg seemed to be equivalent in terms of peak titers and induction of anti-GM2 antibodies. At the 30-μg dose level, 50% of the patients developed complement fixing anti-GM2 antibodies detectable at a serum dilution of 1:10. We conclude that the GM2-KLH/QS21 formulation is more immunogenic than our previous formulation and that 3 μg is the lowest dose that induces consistent, high-titer IgM and IgG antibodies against GM2.

INTRODUCTION

GM2 is a ganglioside expressed on the surface of most melanomas and has been demonstrated to be immunogenic (1, 2). In our previous studies, we demonstrated that melanoma patients who were free of disease after complete surgical resection and who have natural or vaccine-induced antibodies to GM2 have a decreased risk of relapse (3). Immunization with GM2 alone does not induce antibodies (4); induction of optimal immunity against GM2 requires immunization with a potent adjuvant (5). In previous trials, GM2 was mixed with *bacille Calmette-Guérin*, which resulted in short-lived IgM antibodies (titers ≥ 1:80) in approximately 74% of patients, but rarely induced IgG antibodies against GM2 (approximately 10% of patients immunized; Ref. 3). Although IgM antibodies are potent mediators of CMC,[4] we hypothesized that the additional induction of an IgG response against GM2 could result in a more pronounced clinical effect. However, induction of IgG antibodies against carbohydrate antigens such as gangliosides would require a T_H epitope to provide the appropriate signals for immunoglobulin class switching.

To address this issue, GM2 was conjugated to KLH, a carrier protein known to provide T-cell help and administered with adjuvant QS21, a saponin fraction extracted from the bark of the South American tree *Quillaja saponaria Molina* (6). In two pilot studies using GM2 doses of 70 μg, this formulation resulted in high-titer IgG antibodies against GM2 (5, 7). Both IgM and IgG antibodies reacted with GM2$^+$ tumor cells by flow cytometry and induced complement-mediated lysis (8). In these two trials, 32 (76%) of 42 patients developed IgG antibodies against GM2 at titers ≥1:80 when doses of QS21 ≥100 μg were used. Thus, IgG antibodies could consistently be induced against GM2.

The objective of the current trial was to determine the minimal dose of GM2-KLH required for a consistent, high-titer IgM and IgG antibody response. This is one of the first dose-response studies carried out in patients receiving a defined cancer vaccine and identifies a dose that is appropriate for future Phase III trials.

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[4] The abbreviations used are: CMC, complement-mediated cytotoxicity; KLH, keyhole limpet hemocyanin; AUC, area under curve; LDH; lactate dehydrogenase.

Table 1 Dose levels and formulations of GM2-KLH + QS21 vaccine

Dose level (μg of GM2)	No. of patients immunized
1	5
3	10
10	10
30	20*
70	7
Total	52

* The second 10 patients at the 30-μg dose level received vaccine in which the GM2-KLH and QS21 were vialed separately and mixed just prior to administration.

Table 2 Patient characteristics of 52 patients treated

Stage	
II (>4 mm)	4
III	39
IV	9
Gender	
Male/Female	34/18
Primary site	
Trunk	24
Extremity	20
Head/neck	6
Unknown	2
Median age (range)	60 (26–77)
Median time in months from complete resection until first vaccine (range)	5.7 (2.1–12)

MATERIALS AND METHODS

Vaccine Preparation

GM2-KLH was prepared with GM2 from bovine brain and supplied by Progenics Pharmaceuticals, Inc. (Tarrytown, New York) as described previously (5, 9). QS21 was supplied by Aquila BioPharmaceuticals (Framingham, MA).

In general, the vaccine was formulated in a single vial containing both GM2-KLH and QS21. However, a group of 10 patients immunized at the 30-μg dose level were immunized with GM2-KLH and QS21 vialed separately. For these patients, the GM2-KLH and QS21 were mixed by the pharmacist just prior to administration.

Patient Eligibility

Melanoma patients with American Joint Committee on Cancer stage III or IV, or deep stage II (>4 mm), who were free of disease after complete surgical resection were eligible. All of the pathology was confirmed by the Memorial Hospital Pathology Department. In general, patients were started on vaccine within 10 months of surgical resection, but patients were still eligible even after 10 months if their risk of relapse was felt to be >50%. All of the patients signed written informed consent.

Patients were excluded if their Karnofsky performance status was <80, if they had received systemic therapy or radiotherapy within the previous 6 weeks, or if they had a medical condition that would make it difficult to complete the full course of vaccination or to respond immunologically to the vaccine. Women who were pregnant or breast-feeding were not eligible.

Treatment Plan

This trial was carried out under an IND from the United States Food and Drug Administration. Within 4 weeks of starting vaccinations, patients had a physical exam, chest X-ray or chest CT, complete blood count, and comprehensive chemistry screen. An electrocardiogram was required within 10 months of starting the study.

Vaccines were administered by the Clinical Immunology nurses (Clinical Immunology Service, Memorial Sloan-Kettering Cancer Center) as a s.c. injection (final volume, 0.75 ml). Vaccinations were administered on weeks 1, 2, 3, 4, 12, 24, and 36.

This study was designed to compare the immunological effects of different doses of GM2-KLH vaccine. Groups of 5–10 patients were accrued to each of five vaccine dose levels in which the GM2-KLH concentration was adjusted to deliver a GM2 dose of 1, 3, 10, 30, or 70 μg (Table 1). All of the vaccinations contained 100 μg of QS21. Subsequently, the vaccine formulation was changed so that the GM2 KLH and QS21 were prepared in separate vials and mixed just prior to vaccine administration. Using this "two-vial system," an additional 10 patients were immunized at the 30-μg dose level.

Treatment Evaluation

Serological Analysis. Serum was collected immediately prior to each vaccination (including pretreatment), and on weeks 6, 13, 18, 26, 30, 38, and 42. In addition, serum was collected 3 and 6 months after the 7th and final vaccination. Anti-GM2 antibodies were measured using an ELISA method in which GM2 ganglioside is adsorbed to 96-well polystyrene microtiter plates. The remaining binding sites on the plate were blocked by PBS/casein/Tween 20 buffer. Serially diluted patient sera or controls were added, and bound antibody was detected using a goat antihuman IgM or IgG antibody (heavy-chain-specific) conjugated to alkaline phosphatase. Plates were developed using *p*-nitrophenyl phenol substrate, and absorbance was read at 405 nm with a correction of 620 nm. Antibody titer was defined as the highest dilution of patient serum yielding a corrected absorbance of 0.1. Pooled human serum from previously vaccinated patients with a known anti-GM2 antibody titer or pooled normal human serum with no anti-GM2 reactivity were used as positive and negative controls, respectively. A positive serological response was defined as an anti-GM2 titer ≥ 1:80 observed at two or more time points.

The antibody titers plotted *versus* time were also analyzed as the AUC using Prism version 2.01 software (Graph Pad Software, Inc., San Diego, CA). The AUC of the antibody response was considered to represent the overall exposure to anti-GM2 IgG or IgM over time.

CMC Assay. CMC assays were performed by the LDH release method (Boehringer-Mannheim). SK-MEL31 (GM2-positive) or SK-MEL24 (GM2-negative) were plated in 96-well tissue culture plates and incubated at 37°C in a humidified CO_2 incubator. The medium was removed, and plain DMEM containing human serum complement standard (Sigma Chemical Co., St. Louis, MO) was added along with the pre- or postim-

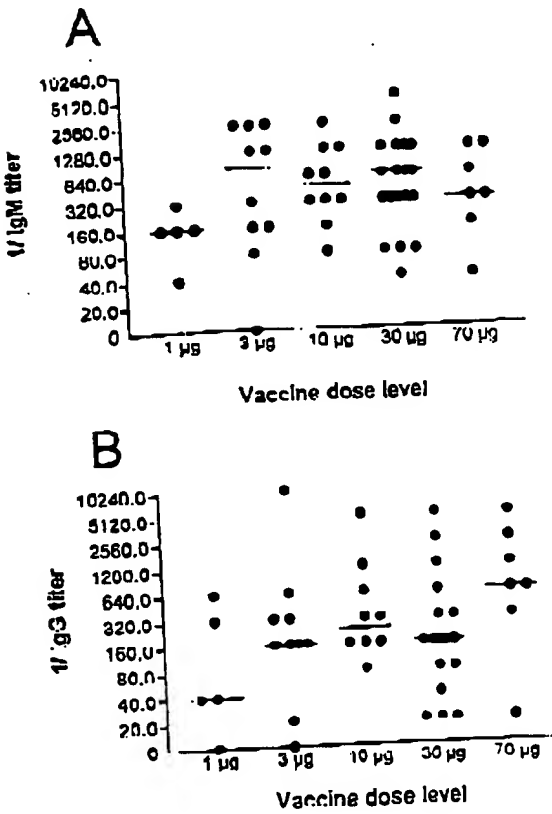

Fig. 1 Peak anti-GM2 antibody titers in patients immunized with GM2-KLH + QS21 at GM2 doses of 1, 3, 10, 30, or 70 µg. *Each dot*, a single patient. The *horizontal lines*, the median peak titers for each dose level. *A*, peak IgM titers; *B*, peak IgG titers.

munization serum to be tested was duplicate wells. The postimmunization serum tested was the serum sample showing the highest IgM anti-GM2 titers for each patient. Both the complement and serum were used at a final dilution of 1:10. In positive control wells, 1% NP40 was added to measure maximal release. The plate was returned to the incubator for 16 h. The supernatants were removed and transferred to a 96-well ELISA plate for analysis. LDH substrate/catalyst was added, and the plate was incubated in the dark at 25°C for 20 min. The plate was read on a spectrophotometer at 492 nm. Each patient's preimmune CMC reading served as the control for the postimmune CMC result. Percent-specific lysis against each cell line was calculated as follows:

$$\frac{\text{(Postimmune serum LDH release} - \text{preimmune serum LDH release)}}{\text{NP40 LDH release}}$$

Clinical Evaluation. Patients were evaluated clinically at Memorial Hospital on weeks 12, 24, and 36 and on three months after the 7th vaccination. A chest X-ray, complete blood

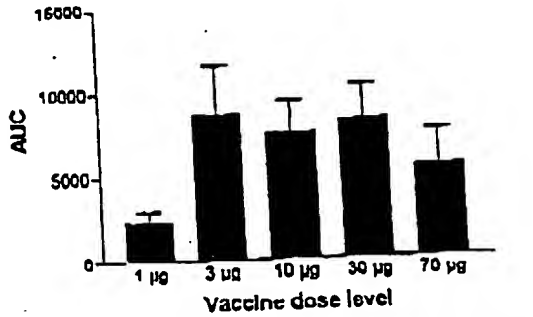

Fig. 2 AUC analysis for the IgM anti-GM2 responses at each dose level. The AUC was calculated for each patient up to week 30. *Height of the columns*, the mean (± SE) AUC for each dose level.

count and comprehensive screening profile were repeated at the time of the 5th and 7th vaccination; an electrocardiogram was repeated at the time of the 7th vaccination. Toxicity was scored using standard criteria (10).

RESULTS

Patient Characteristics. Fifty two patients were entered on this trial between January 1995 and April 1996 (Table 2). There were 34 men and 18 women. Most (75%) of the patients had stage III melanoma; 8% had deep stage II, and 17% had stage IV. The patients had been free of disease for a median of 5.7 months before beginning the trial.

Serological Results. Applying rigorous definitions of response (defined in "Materials and Methods") 88% of the patients immunized in this study developed an IgM response against GM2; 71% developed an IgG response. Fig. 1 shows the peak anti-GM2 titers attained at each dose level. For IgM, the median peak titers ranged from 1:160 to 1:800; for IgG the median peak titers ranged from 1:40 to 1:640. When comparing the incidence of nonresponding patients (peak titers ≤1:40) for IgM and IgG at each of the dose levels, we found no difference for the IgM response ($P = 0.73$; χ^2) or IgG response ($P = 0.19$; χ^2). From the exploratory analysis, it appeared that there were fewer IgG responses at the 1-µg dose level.

An AUC analysis was performed for both IgM and IgG anti-GM2 responses on each patient until week 30, and the mean AUCs at each dose level were compared. For the IgM anti-GM2 response, the mean AUC at the 1-µg dose level was lower than the mean AUC at any of the other dose levels (Fig. 2). The mean AUC for the IgG response was also lower in patients treated at the 1-µg dose level compared with the mean AUCs at the other dose levels (data not shown), but this difference was not statistically significant. There were no differences in the AUC for the other dose levels.

Given that the 1-µg dose level seemed to have a lower incidence of inducing IgG against GM2 and a lower mean AUC for the IgM response, we concluded that the 1-µg dose level was less immunogenic than the other dose levels. As a result, we focused on the 3-, 10-, 30-, and 70-µg dose levels.

Fig. 3 illustrates the median anti-GM2 IgM and IgG titers for patients immunized at the 3-, 10-, 30-, and 70-µg dose

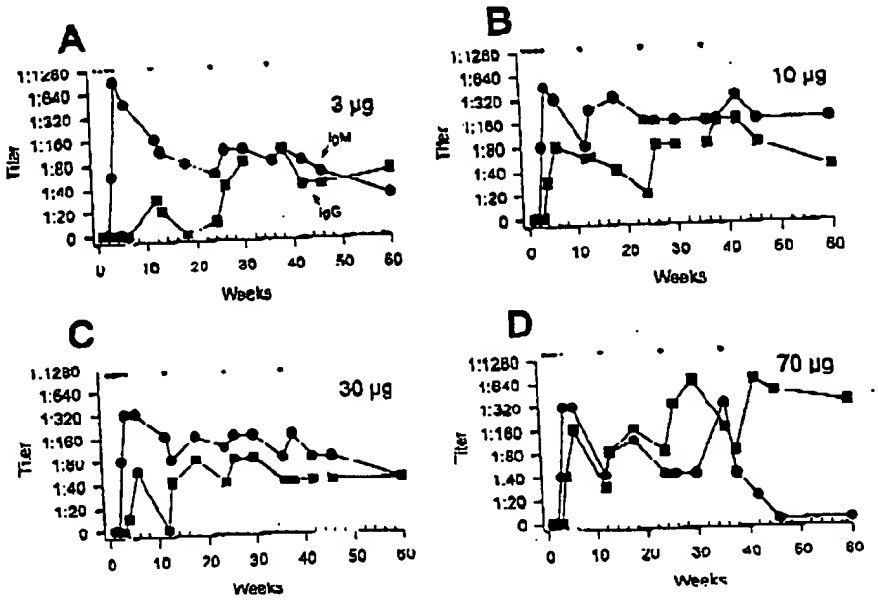

Fig. 3 Median anti-GM2 antibody titers in patients immunized with GM2-KLH + QS21 at GM2 doses of 3 μg (*A*), 10 μg (*B*), 30 μg (*C*), or 70 μg (*D*). IgM titers (●) and IgG titers (■) are shown separately at each dose level. ▾, administration of vaccine.

levels. At these four dose levels, there was a consistent IgM response followed by an IgG response. Both the IgM and IgG responses were sustained for months after the final immunization. At week 60 (5½ months after the last immunization), serum was available on 20 patients who had developed an IgM response and 19 patients who had developed an IgG response. Analysis of these sera showed that the IgM response persisted in 45% of the cases; the IgG response persisted in 53% of the cases (data not shown). This demonstrates that, in one-half of the patients who developed anti-GM2 antibodies, the antibody response persisted for at least 5½ months.

Most of the patients immunized on this trial received vaccine that had been formulated in one vial (*i.e.*, GM2-KLH and QS21 were stored together). However, 10 of the 20 patients immunized at the 30-μg dose level received vaccine formulated in two vials because we obtained evidence that the stability of the vaccine was enhanced if the GM2-KLH and QS21 were stored in separate vials and mixed just prior to vaccine administration. We compared the anti-GM2 response induced in patients immunized with the single-vial *versus* the two-vial formulation at the 30-μg dose level (Fig. 4). The median IgM titers were similar in the two groups; the median IgG titers were slightly lower in the group receiving vaccine formulated in two vials. All of the patients immunized with the single-vial formulation developed anti-GM2 antibodies, and only one patient immunized with the two-vial formulation failed to develop anti-GM2 antibodies. We conclude that there was no difference in the immunogenicity between the one-vial and the two-vial formulations.

CMC. Sera from 18 of the 20 patients treated at the 30 μg dose level were available to be tested for the ability to bind melanoma cells and to fix the complement (Fig. 5). In 9 of the 18 patients, the postvaccination sera showed an increase in

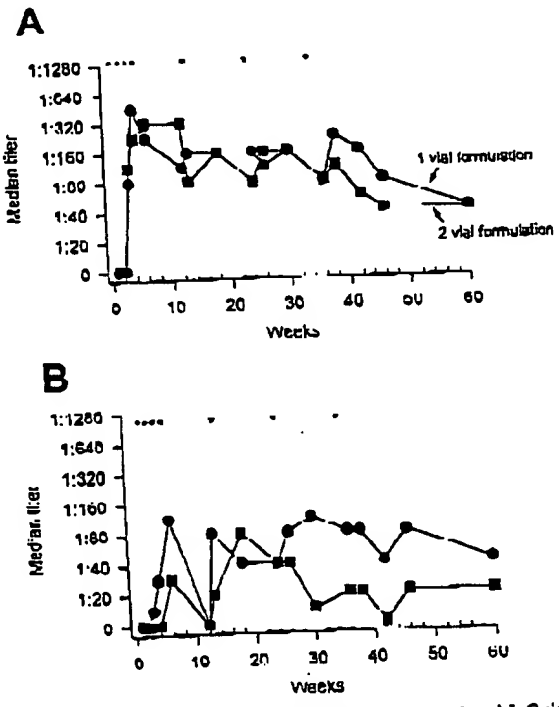

Fig. 4 Comparison of median anti-GM2 IgM titers (*A*) and IgG titers (*B*) among patients immunized at the 30-μg dose level. ●, patients immunized with vaccine formulated in a single vial; ■, patients immunized with vaccine formulated in 2 vials, in which the GM2-KLH and QS21 vialed separately; ▾, administration of vaccine.

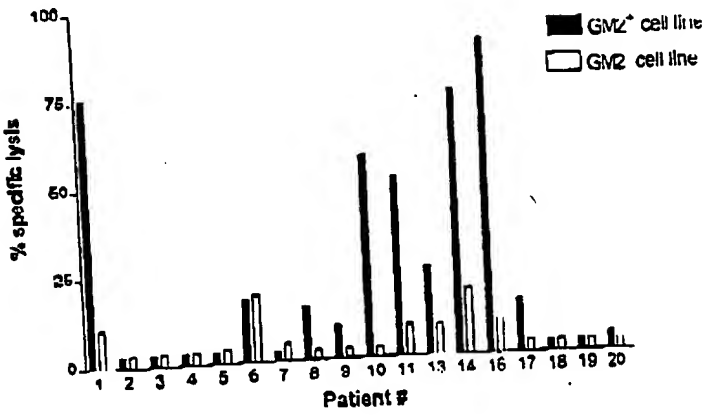

Fig. 5 CMC of sera from patients immunized at the 30 μg dose level. ■, the increase of CMC against a $GM2^+$ cell target in postvaccination sera compared with pretreatment sera. □, the increase of CMC against a $GM2^+$ cell target in postvaccination sera compared with pretreatment sera. Data for patients 12 and 15 are not available.

CMC compared to pretreatment that was specific for the $GM2^+$ cell target. In the remaining nine patients, there was either no increase in CMC compared with pretreatment levels (patients 2, 3, 4, 5, 7, 18, 19, and 20) or the increase was not specific for GM2 (patient 6). Induction of complement-fixing activity correlated with a peak IgM anti-GM2 titer of 1:640. All of the nine patients demonstrating CMC activity in their serum had peak IgM anti-GM2 titers ≥ 1:640 as opposed to only two of nine patients without CMC activity ($P = 0.002$; Fisher's exact test).

Toxicity. Virtually all of the patients experienced inflammation and/or pruritis at the site of injection attributed to the known effects of the QS21 adjuvant (7). Other common side effects were: (*a*) fever (71%); (*b*) mild fatigue (44%) and flu-like symptoms (58%); (*c*) chills (29%); and (*d*) myalgias (48%). These were self-limiting, never more severe than grade 2, and rarely lasted more than 24 h. Headache was seen in 66% of the patients and was grade 1–2 except in one patient with a grade-3 headache. These toxicities were felt to be due largely to QS21, which is consistent with the observation that there was no correlation between vaccine dose and toxicity. Grade 3 or 4 toxicity possibly related to vaccine occurred in four patients. One patient developed transient dyspnea, which resolved spontaneously. Another patient reported 2–3 days of severe dizziness, which also resolved spontaneously. One patient developed atrial flutter while on the study and required treatment. A fourth patient, with a history of migraine headaches, reported a grade 3 headache associated with vaccine therapy. No patient was taken off study because of toxicity.

DISCUSSION

The current trial confirms that vaccinating melanoma patients with GM2-KLH + QS21 induces both IgM and IgG antibodies against GM2. We observed that 88% of patients developed IgM anti-GM2 antibodies and 71% developed IgG anti-GM2 antibodies. This compares almost exactly with the immunological results observed in our previous pilot trials (5, 7). Because the previous trials used vaccine produced at Memorial Sloan-Kettering Cancer Center and the current trial used vaccine produced by Progenics Pharmaceuticals, Inc., this demonstrates that subsequent lots of the vaccine can be produced successfully and that the immunogenicity is reproducible. The results also show that the vaccine can be formulated either with QS21 or vialed separately and mixed with QS21 just prior to administration. We favor formulating GM2-KLH and QS21 in separate vials because of improved stability.

This is one of the first cancer vaccine trials to explore dose-response effects using a defined antigen. Our previous trials used GM2-KLH at a GM2 dose of 70 μg and demonstrated that all of the patients developed IgM anti-GM2 and 76% developed IgG anti-GM2. In this current trial, we have explored GM2 doses of 1, 3, 10, 30, and 70 μg. We conclude that the immunogenicity of GM2-KLH at a GM2 dose of 1 μg is suboptimal based on the fact that the 1-μg dose was less likely to induce IgG anti-GM2 antibodies. The mean AUC for the anti-GM2 IgM antibody responses was also lowest for the 1-μg dose level, which implies that this dose resulted in the lowest level of tumor-cell exposure to anti-GM2 antibody. At the higher vaccine doses (3, 10, 30, or 70 μg), there was no apparent difference in the immunogenicity of the vaccine. Peak titers, AUC, antibody responses over 60 weeks, and percent of nonresponding patients were similar at the 3-, 10-, 30-, and 70-μg dose levels.

In patients immunized at the 30-μg dose level, 50% of the patients developed antibodies that fixed complement and resulted in CMC against $GM2^+$ melanoma. CMC activity correlated with peak IgM anti-GM2 titers ≥1:640. This demonstrates that immunization induced anti-GM2 antibodies capable of binding cell-surface GM2 and mediating effector functions.

In at least one-half of the patients, the anti-GM2 antibody response persisted for more than 5½ months. This is consistent with the notion that the KLH carrier protein provides sufficient T-cell help to induce a more prolonged antibody response against GM2. It is also important to note that patients at the 70-μg dose level received a 23-fold higher KLH dose compared with patients at the 3-μg dose level, and that this was not associated with any excessive toxicity or decreased immunogenicity. This is reassuring as we consider construction of multivalent vaccines containing 4 or 5 antigens conjugated to KLH. Our results suggest that these higher

total KLH doses will neither be more toxic nor lead to diminished immunogenicity.

These studies provide a basis for additional trials with GM2-KLH + QS21. Future clinical trials will examine the effects of IFN-α on the anti-GM2 response induced by GM2 KLH + QS21, the immunogenicity of GM2-KLH + QS21 combined with GD2-KLH, and a Phase III trial comparing GM2-KLH + QS21 to IFN-α for the ability to prevent recurrence of melanoma in stage III patients. For these trials, a vaccine dose ≥3 μg of GM2 should be used.

ACKNOWLEDGMENTS

We are grateful to the Immunology Nurses at Memorial Sloan Kettering Cancer Center who dedicated much effort to the implementation of this protocol.

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Exhibit F

Sheet 1 of 1

Form PTO-1449 U.S. Department of Commerce Patent and Trademark Office	Atty. Docket No. 1747/43016-A-PCT-US	Serial No. 08/196,154
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		5 6 1 6 4 7 7	4/1/97	Price	Exhibit	G	July 7, 1994

FOREIGN PATENT DOCUMENTS

		Document Number	Date	Country	Class	Subclass	Translation Yes	Translation No

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

EXAMINER	DATE CONSIDERED

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Exhibit G

US005616477A

United States Patent [19]

Price

[11] Patent Number: 5,616,477

[45] Date of Patent: Apr. 1, 1997

[54] FUSION PROTEINS COMPRISING GM-CSF AND ANTIGENS AND THEIR EXPRESSION IN YEAST

[75] Inventor: Virginia L. Price, Seattle, Wash.

[73] Assignee: Immunex Corporation, Seattle, Wash.

[21] Appl. No.: 641,704

[22] Filed: May 2, 1996

Related U.S. Application Data

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[51] Int. Cl.[6] C12N 1/19; C12N 15/27; C12N 15/62

[52] U.S. Cl. 435/69.5; 435/69.7; 435/252.3; 435/320.1; 536/23.4

[58] Field of Search 435/69.5, 69.7, 435/252.3, 320.1; 536/23.4

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0604727A1 7/1994 European Pat. Off. .

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Primary Examiner—John Ulm

Attorney, Agent, or Firm—Patricia Anne Perkins; Charlene Launer

[57] ABSTRACT

Novel fusion proteins that enhance the immune response of an antigen are efficiently expressed and secreted by yeast host cells. The fusion proteins are recombinantly made by fusing the 3'-end of mature GM-CSF DNA sequence to the 5'-end of an antigen DNA sequence with or without a linker sequence. Methods of expression in yeast cells are provided.

23 Claims, No Drawings

FUSION PROTEINS COMPRISING GM-CSF AND ANTIGENS AND THEIR EXPRESSION IN YEAST

CROSS REFERENCE TO RELATED APPLICATION

This application is a continuation of application Ser. No. 08/271,875, filed Jul. 7, 1994, now abandoned.

BACKGROUND OF THE INVENTION

The present invention relates generally to the construction of fusion proteins that enhance the immune response of an antigen and are efficiently expressed and secreted by yeast host cells. More specifically, the invention relates to yeast recombinant expression systems for producing fusion proteins comprising a granulocyte-macrophage colony-stimulating factor (GM CSF) domain fused to a selected antigen domain.

Technologies for the efficient production of large quantities of antigenic proteins for use as immunogens have been sought for many years. Genes encoding protein antigens and fragments of antigens comprising particular epitopes have been expressed in prokaryotic and eukaryotic cell expression systems with varying degrees of success. To elicit an antibody response in animals, administration of adjuvants, repeated administration of the expressed protein, or both often were needed.

Certain materials have been shown to have adjuvant activity, including for example alum, fragments of bacterial membranes, liposomes, coupling a protein of interest to a larger immunogenic protein, RIBI, and TiterMax®. Of all of them, alum is the only adjuvant licensed by the Food and Drug Administration for use in humans. Some investigators have attempted to chemically couple adjuvants to antigens. Such coupling involves harsh treatment and often results in destruction of a portion of the antigen and reduced antigenicity.

Some cytokines, e.g., interleukin-4 (IL-4) and GM-CSF, attract and activate antigen-presenting cells for more efficient presentation of antigens to T cells. These cytokines have been co-administered with antigen to increase antigenic activity. Other studies have shown that the host response to tumor challenge can be increased by inoculation of tumor cells genetically engineered to express particular cytokines, including γ-INF, TNF-α, IL-2, IL-4, IL-6, IL-7, or GM CSF.

Further, Tao and Levy (*Nature*, 362: 755–758 (1993)) created chimeric tumor idiotype/GM-CSF fusion proteins as vaccines for B-cell lymphoma. They created their fusion proteins by constructing plasmids with a coding sequence of a heavy-chain variable region from a mouse B cell tumor inserted upstream of a human IgG1 heavy-chain constant region gene and a restriction site generated next to the last codon of the CH3 exon into which genetic fragments encoding either murine or human GM-CSF were inserted. These heavy chain vectors were then cotransfected with a light chain chimeric constructs into a malignant plasma cell tumor. The proteins made by the transfected cells were tetrameric proteins that were dimeric with respect to GM-CSF.

Research continues toward enhancing the immune response of an antigen. Proteins that have the bioactivity of both cytokines and antigens will provide the advantages of using chemically defined antigenic entities and eliminating the need to separately administer or co-administer cytokines and antigens or inoculate patients with genetically modified living tumor cells.

SUMMARY OF THE INVENTION

Novel fusion proteins comprising either mature murine or human GM-CSF fused to a selected antigen are efficiently expressed in yeast at very high levels, with virtually all the material made being secreted from the yeast. The fusion proteins are created using standard molecular biology techniques to fuse the 3'-end of mature GM-CSF DNA sequence to the 5'-end of an antigen DNA sequence. The GM-CSF DNA sequence is fused to the antigen DNA sequence with or without a linker peptide sequence. DNA encoding the GM-CSF/antigen fusion protein is operably linked to suitable transcriptional or translational regulatory elements. Preferably, the regulatory elements include an ADH2 promoter and a secretion signal is either a yeast α-factor leader or a type I interleukin-1 receptor (IL-1R) signal sequence lacking its native signal peptidase recognition site. Yeast cells transformed with the resulting expression vector are cultivated to express and secrete large quantities of the desired fusion protein, that are recovered from the culture supernatant. These fusion proteins have the biological activity of both GM-CSF and the antigen.

The invention also provides for methods of producing a GM-CSF/antigen fusion protein that has the biological activity of both GM-CSF and the selected antigen. One such method includes ligating the 3'-end of a DNA sequence encoding mature GM-CSF to the 5' end of a DNA sequence encoding a selected antigen; linking the ligated DNA sequences to regulatory elements that are responsible for expression of DNA into a single biologically active protein; inserting the ligated DNA sequence into a yeast host cell, culturing the yeast host cell under conditions promoting expression; and recovering the desired fusion protein from the culture. The regulatory elements preferably include an ADH2 promoter and a secretion signal that is either a yeast α-factor leader or a type I interleukin-1 receptor (IL-1R) signal sequence lacking its native signal peptidase recognition site.

A second method includes culturing a yeast cell transformed with an expression vector comprising a promoter, a sequence encoding GM-CSF fused in frame to the 3'-end of a DNA sequence encoding an antigen, and a stop codon under conditions that promote expression of said fusion protein; and recovering the desired fusion protein from said culture. Preferably, the promoter is an ADH2 promoter. Further, the expression vector preferably includes a secretion signal is either a yeast α-factor leader or a type I interleukin-1 receptor (IL-1R) signal sequence lacking its native signal peptidase recognition site.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "GM-CSF" refers to proteins having amino acid sequences that are substantially similar to the native human granulocyte-macrophage colony-stimulating factor amino acid sequences (e.g., ATCC 53157) and that are biologically active in that they are capable of binding to GM-CSF receptors, transducing a biological signal initiated by binding GM-CSF receptors, or cross-reacting with antibodies raised against GM-CSF. Such sequences are disclosed, for example, by Anderson et al. in *Proc. Nat'l. Acad. Sci. USA* 82: 6250 (1985). The term "GM-CSF" also

includes analogs of GM-CSF molecules that exhibit at least some biological activity in common with native human GM-CSF. Exemplary analogs of GM-CSF are disclosed in EP Publ. No. 212914 (U.S. Ser. No. 06/763,130), which describes GM-CSF analogs having KEX2 protease cleavage sites inactivated so as to increase expression of GM-CSF in yeast hosts, and in WO Publ. No. 89/03881 (U.S. Pat. No. 5,032,676), which describes GM-CSF analogs having various glycosylation sites eliminated.

The term "antigen" refers to a tumor antigen or foreign protein that induces the formation of antibodies that interact specifically with it. Each antigen may contain more than one site (antigenic determinant) capable of binding to a particular antibody. Thus, an antigen can cause the production of a number of antibodies with different specificities. A "foreign protein" refers to a protein other than one encoded by or derived from the human genome; e.g., a microbial or viral protein or parasite protein.

"Biologically active" as used herein means that a particular molecule shares sufficient amino acid sequence similarity with native forms so as to be capable of binding to native receptors, transmitting a stimulus to a cell, or cross-reacting with antibodies raised against the particular molecule.

As used herein, the term "fusion protein" refers to an antigen fused to the C-terminal portion of GM-CSF. Specifically, the fusion proteins of the present invention have a formula selected from the group consisting of

$$R_1\text{-}L\text{-}R_2 \text{ or } R_1\text{-}R_2$$

wherein R_1 is GM-CSF; R_2 is an antigen; and L is a peptide linker sequence. The antigen is linked to GM-CSF in such a manner as to produce a single protein that retains the biological activity of the native antigen and murine GM-CSF. Unless otherwise specified, the term "GM-CSF/antigen" refers to fusion protein with a peptide linker sequence added.

"Recombinant expression vector" refers to replicable DNA constructs that contain a synthetic or cDNA-derived DNA sequence encoding one of the above-described fusion proteins, operably linked to suitable transcriptional or translational regulatory elements. Examples of genetic elements having a regulatory role in gene expression include transcriptional promoters, operators or enhancers, a sequence encoding suitable mRNA ribosomal binding sites, and appropriate transcription and translation initiation and termination sequences. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants may additionally be incorporated. The regulatory elements employed in the expression vectors that are conventionally used to express recombinant proteins in *S. cerevisiae* may be used. Regulatory elements for use in yeast expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it may include an N-terminal methionine residue. This residue may optionally be subsequently cleaved from the expressed recombinant protein to provide a final product.

DNA regions are operably linked when they are functionally related to each other. A DNA sequence encoding a fusion protein is operably linked to one or more of the above-described regulatory elements when the fusion protein DNA sequence is transcribed, or the resulting mRNA is translated, under the control of the regulatory element(s). For example, DNA for a signal peptide (secretory leader) is operably linked to DNA for a polypeptide if it is expressed as a precursor that participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of secretory leaders, contiguous and in reading frame.

"Transformed host cells" are cells that have been transformed or transfected with foreign DNA using recombinant DNA techniques. In the context of the present invention, the foreign DNA includes a sequence encoding the inventive fusion protein. Host cells may be transformed for purposes of cloning or amplifying the foreign DNA, or may be transformed with an expression vector for production of the fusion protein under the control of appropriate promoters. Examples of appropriate cloning and expression vectors for use with yeast hosts are described by Powels et al. in *Cloning Vectors: A Laboratory Manual*, Elsevier, N.Y. (1985). Cell-free translation systems also could be employed to produce fusion protein using RNAs derived from the DNA constructs of the present invention.

A "DNA sequence" refers to a DNA polymer, in the form of a separate fragment or as a component of a larger DNA construct, that has been derived from DNA isolated at least once in substantially pure form (i.e., free of contaminating endogenous materials) and in a quantity or concentration enabling identification, manipulation, and recovery of its component nucleotide sequences by standard biochemical methods such as those outlined by Sambrook et al. in *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989). Such sequences are preferably provided in the form of an open reading frame uninterrupted by internal nontranslated sequences, or introns, that are typically present in eukaryotic genes. Sequences of non-translated DNA may be present 5' or 3' from an open reading frame, where the same do not interfere with manipulation or expression of the coding regions.

"Nucleotide sequence" refers to a heteropolymer of deoxyribonucleotides. DNA sequences encoding the proteins provided by this invention may be assembled from cDNA fragments and short oligonucleotide linkers, or from a series of oligonucleotides, to provide a synthetic gene that is capable of being expressed in a recombinant transcriptional unit.

The term "heterologous protein" as used herein indicates that the protein to be expressed is not naturally expressed in the yeast host cell.

Fusion Proteins and Analogs

The present invention provides fusion proteins comprising a murine or human GM-CSF domain and a selected antigen. The fusion proteins also may include a linker peptide between the GM-CSF and the antigen.

The primary amino acid structure may be modified by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like, or by creating amino acid sequence mutants. Covalent derivatives are prepared by linking particular functional groups to amino acid side chains or at the N- or C-termini. Other derivatives of the fusion protein within the scope of this invention include covalent or aggregative conjugates of the fusion protein with other proteins or polypeptides.

Peptides also may be added to facilitate purification or identification of GM-CSF/antigen fusion proteins (e.g., poly-His). The amino acid sequence of the fusion protein also can be linked to the peptide disclosed by Hopp et al. in

BioTechnology 6: 1204, (1988). The latter sequence is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid assay and facile purification of expressed recombinant protein.

Derivatives and analogs may be obtained by mutations of the fusion protein. A derivative or analog is a polypeptide in which the GM-CSF or antigen domains are substantially homologous to the native GM-CSF (e.g., ATCC 53157) and the native antigen of choice but have an amino acid sequence difference attributable to a deletion, insertion or substitution.

Biocquivalent analogs of GM-CSF or antigen domains to be incorporated into the fusion proteins may be constructed by making various substitutions of amino acid residues or sequences, or by deleting terminal or internal residues or sequences not needed for biological activity. For example, Cys residues can be deleted or replaced with other amino acids to prevent formation of incorrect intramolecular disulfide bridges upon renaturation. Other approaches to mutagenesis involve modification of dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present. Generally, substitutions are made conservatively by substituting an amino acid having physiochemical characteristics resembling those of the native residue.

Mutations in nucleotide sequences constructed for expression of analogs must, of course, preserve the reading frame phase of the coding sequences and preferably will not create complementary regions that could hybridize to produce secondary mRNA structures such as loops or hairpins that would adversely affect translation of the GM-CSF/antigen mRNA. Although a mutation site may be predetermined, it is not necessary that the nature of the mutation per se be predetermined. For example, in order to select for optimum characteristics of mutants at a given site, random mutagenesis may be conducted at the target codon and the expressed mutants screened for the desired activity.

Not all mutations in nucleotide sequences that encode fusion proteins comprising GM-CSF and the antigen of choice will be expressed in the final product. For example, nucleotide substitutions may be made to enhance expression, primarily to avoid secondary structure loops in the transcribed mRNA (see EPA 75,444A, incorporated herein by reference), or to provide codons that are more readily translated by the selected host, e.g., the well-known *E. coli* preference codons for *E. coli* expression.

Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion. In addition, the polymerase chain reaction (PCR) can be used to generate mutant DNA sequences.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Exemplary methods of making the alterations set forth above are disclosed by Walder et al. in *Gene* 42: 133 (1986); Bauer et al. in *Gene* 37: 73 (1985); Craik in *BioTechniques* p. 12-19 (January 1985); Smith et al. in *Genetic Engineering: Principles and Methods*, Plenum Press (1981); and U.S. Pat. Nos. 4,518,584 and 4,737,462, and are incorporated by reference herein.

Source of Recombinant Fusion Proteins Comprising GM-CSF and an Antigen

A DNA sequence encoding a fusion protein is constructed using recombinant DNA techniques to assemble separate DNA sequences encoding GM-CSF and the antigen into an appropriate expression vector. The 3' end of a DNA sequence encoding mature GM-CSF is ligated, with or without a peptide linker, to the 5' end of the DNA sequence encoding a biologically active antigen of choice, so that the reading frames of the sequences are in phase to permit mRNA translation of the two DNA sequences into a single biologically active fusion protein. The resulting protein is a GM-CSF/antigen fusion protein.

In preferred aspects of the present invention, a peptide linker sequence is incorporated into the fusion protein construct by well-known standard molecular biology techniques (e.g., PCR). The linker sequence is used to separate GM-CSF and the antigen domains by a distance sufficient to ensure that each domain properly folds into its secondary and tertiary structures. Suitable peptide linker sequences (1) will adopt a flexible extended conformation, (2) will not exhibit a propensity for developing an ordered secondary structure that could interact with the functional GM-CSF and antigen domains, and (3) will have minimal hydrophobic or charged character that could promote interaction with the functional protein domains. Typical surface amino acids in flexible protein regions include Gly, Asn and Ser. Virtually any permutation of amino acid sequences containing Gly, Asn and Ser would be expected to satisfy the above criteria for a linker sequence. Other near neutral amino acids, such as Thr and Ala, also may be used in the linker sequence. Thus, amino acid sequences useful as linkers of GM-CSF and antigen include the $Gly_4SerGly_5Ser$ linker (SEQ ID NO 1) used in U.S. Pat. No. 5,108,910 or a series of four (Ala Gly Ser) residues (SEQ ID NO 2). Still other amino acid sequences that may be used as linkers are disclosed in Maratea et al., *Gene* 40: 39–46 (1985); Murphy et al., *Proc. Nat'l. Acad. Sci. USA* 83: 8258–62 (1986); U.S. Pat. No. 4,935,233; and U.S. Pat. No. 4,751,180.

The length of the peptide linker sequence may vary without significantly affecting the biological activity of the fusion protein. In one preferred embodiment of the present invention, a peptide linker sequence length of about 12 amino acids is used to provide a suitable separation of functional protein domains, although longer linker sequences also may be used. The linker sequence may be from 1 to 50 amino acids in length. In the most preferred aspects of the present invention, the linker sequence is from about 1-20 amino acids in length. In the specific embodiments disclosed herein, the linker sequence is from about 5 to about 15 amino acids, and is advantageously from about 10 to about 15 amino acids. Peptide linker sequences are unnecessary where the proteins being fused have non essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference.

The ligated DNA sequences are operably linked to suitable transcriptional or translational regulatory elements. The regulatory elements responsible for expression of DNA are located only 5' to the GM-CSF cDNA fragment, while these regulatory elements or stop codons that would prevent read-through to the antigen DNA fragment, are not present on the GM-CSF fragment. Conversely, regulatory elements are not present on the antigen DNA fragment while stop codons required to end translation and transcription termination signals are present only 3' to the antigen DNA fragment.

Expression of Recombinant Fusion Proteins Comprising GM-CSF and an Antigen

The present invention provides for recombinant expression vectors that include synthetic or cDNA-derived DNA

fragments encoding fusion proteins comprising GM-CSF and an antigen of choice or bioequivalent analogs operably linked to suitable transcriptional or translational regulatory elements. The regulatory elements include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences that control the termination of transcription and translation, as described in detail below. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants may additionally be incorporated.

The fusion protein vectors are transformed or transfected into host cells. Transformed host cells ordinarily express the desired fusion protein, but host cells transformed for purposes of cloning or amplifying DNA do not need to express the protein. Expressed fusion protein will generally be secreted into the culture supernatant. The present invention provides for expression of the inventive fusion proteins in yeast under the control of appropriate regulatory elements.

Our recombinant fusion proteins are expressed in yeast hosts, preferably from the Saccharomyces species, such as *S. cerevisiae*. Yeast of other genera such as Pichia or Kluyveromyces also may be employed. Those skilled in the art will readily see that other expression systems, such as mammalian and insect expression systems with appropriate regulatory elements, also can be used to express the desired fusion protein. Secretion of the desired protein from the yeast cells is advantageous since the desired protein is recovered from the culture supernatant rather than from the complex mixture of proteins that results when yeast cells are disrupted to release intracellular proteins. Secretion also reduces the deleterious (e.g., toxic) effect that intracellular accumulation of a given foreign protein may have on the host cell.

The yeast *S. cerevisiae* is often used for the expression and secretion of heterologous proteins. Efficient, high-level secretion of a cerevisiae protein from yeast requires not only efficient transcription and translation of the mRNA, but at the posttranslational level, efficient processing of the leader sequence that directs secretion and routing through the secretory pathway. Efficient processing of a signal (pre) or additional pro sequences used to direct secretion first requires enzymatic cleavage at the signal peptidase site and, if present, additional cleavage at the 3' end of the pro sequence (the KEX2 site for the alpha-factor leader). If the signal sequence fails to be cleaved off in the endoplasmic reticulum, the protein does not continue through the secretory pathway. Similarly, if the additional processing site(s) at the 3' end of a pro region are not cleaved, secretion is either greatly inhibited, or if it does occur, the desired protein has additional amino acids at the N-terminus. See, e.g., Brake et al., *Proc. Nat'l. Acad. Sci.*, 81: 4642-4646 (1984). The particular amino acid sequences that are present 3' to these cleavage sites have an effect on the ability of the sites to be processed. Some heterologous amino acid sequences fused 3' to a secretion signal cause inefficient cleavage, thus poor secretion while others allow efficient cleavage, thus good secretion. Human and murine GM-CSF are examples of heterologous proteins that can be secreted from yeast at very high levels, with virtually all the material made being secreted from the yeast.

We have found that the presence of the N-terminal sequences of GM-CSF fused 3' of either the signal peptidase site present on the type I IL-1R signal sequence (described below) or the KEX2 site present on the α-factor pro region allow efficient processing of these signals. With any different heterologous protein placed immediately 3' to the processing signals, it is unknown whether there would be efficient processing, thus secretion. If a heterologous cDNA were fused 3' to the GM-CSF gene, the junction between the signal or pro processing sites and GM-CSF would be maintained and one would expect to achieve efficient processing and secretion of the fusion molecule. Any additional benefits of the GM-CSF protein and its ability to "route" through the secretory system would be maintained, too. The fusion to GM-CSF thus eliminates one of the key variables in secretion of heterologous proteins in yeast. Such a fusion system for expression in yeast is ideally suited to the fusion of peptides (5–50 amino acids) or relatively small proteins of about a molecular mass of 20,000 daltons or less to GM-CSF. For the expression of antigens larger than 30,000 daltons, those of ordinary skill in the art can design an analogous system for expression in mammalian or insect cells or other yeasts.

S. cerevisiae strain YIMX9 is particularly useful as a host cell of the expression of GM-CSF/antigen fusion proteins. The YIMX9 strain was generated and isolated as follows. The procedures are generally as described in Rose et al., *Methods in Yeast Genetics, A Laboratory Course Manual*, Cold Spring Harbor Press, Cold Spring Harbor, N.Y., pages 13–15 (1990). *S. cerevisiae strain* XV617 1 3B [a, his6, leu2-1, trp1-1, ura3, ste5] was obtained University of Washington, Department of Genetics Yeast Strain Bank, Seattle, Wash. A fresh overnight culture of XV617-1-3B transformed with a recombinant expression vector was grown in YEPG (1% yeast extract, 2% peptone, 2% glucose) to a cell density of about $1–2\times10^8$ cells/ml. The vector encoded a reporter protein that is not well secreted from this strain. The culture was diluted to 5×10^5 cells/ml in KH_2PO_4, pH 7.0, 10 mls total volume. 0.45 ml of the mutagen ethylmethane sulfonate (EMS, available from Sigma Chemical Co., St. Louis, Mo.) was added, and the culture was incubated at 30° C. for 30 minutes. Cells were then plated at a density of 500–1000 cells/plate on YNB -trp medium (0.67% yeast nitrogen base, 2% glucose, amino acids minus tryptophan at approximately 20 μg/ml).

Colonies were screened for secretion of the reporter protein using an antibody immunoreactive with the reporter protein. Positive colonies were detected by binding of the antibody to secreted product on nitrocellulose filters. A mutant isolated from this screening process was designated YIMX1. Strain YIMX1 was crossed to strain X2181-1B [a, upl-1, gal1, ade1, his2], obtained from the Yeast Genetic Stock Center, University of California, Berkeley, Calif., to create the diploid strain designated YIMX2. This diploid is heterozygous at the mutant locus (an unidentified locus that allows improved secretion of the reporter protein). The mutation of interest was shown to be recessive in that strain. YIMX2 did not exhibit the property of better secretion of the reporter protein. For this reason, UV mutagenesis was performed on YIMX2 to induce homozygosis at the mutant locus (a crossing-over event that would result in information from one chromosome replacing that on the homologous chromosome).

YIMX2 was transformed with the reporter-encoding expression vector employed in the first mutagenesis procedure. The UV source was a Stratalinker® UV Crosslinker (Stratagene Cloning Systems, LaJolla, Calif.), that emits about 0.67 mjoules per second. YNB -trp plates spread with $0.5–1\times10^3$ colonies per plate were irradiated for 12–15 seconds. Colonies were screened as above for increased secretion of the reporter protein. A strain demonstrating increased secretion of the reporter protein was isolated and designated YIMX9. A sample of the isolated mutant strain *S. cerevisiae* YIMX9 was deposited under the terms of the

Budapest Treaty with the American Type Culture Collection in Rockville, Md., and assigned accession number ATCC 74274.

Another particularly useful host cell is the *S. cerevisiae* strain designated XV2181 (a/α, upl, V. Price et al., *Gene*, 55: 287–293 (1987)). XV2181 was formed by mating the above-described strains XV617-1-3B and X2181-1B.

Appropriate cloning and expression vectors for use with yeast are described herein and by Pouwels et al. in *Cloning Vectors: A Laboratory Manual*, Elsevier, N.Y. (1985). Expression vectors generally comprise one or more phenotypic selectable markers (e.g., a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement) and an origin of replication recognized by the intended host cell to ensure amplification within the host. Yeast vectors commonly contain an origin of replication from the 2 μm yeast plasmid or an autonomously replicating sequence (ARS), a promoter, DNA encoding the fusion protein, sequences for polyadenylation and transcription termination and a selectable marker. Some yeast vectors will include an origin of replication and selectable marker permitting transformation of both yeast and *E. coli*, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, that provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, and a promoter derived from a highly expressed yeast gene to induce transcription of a structural sequence downstream. The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoter sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255: 2073 (1980)) or glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* 7: 149 (1968), and Holland et al., *Biochem.* 17: 4900 (1978)), such as the ADH2 promoter (Russell et al. in *J. Biol. Chem.* 258: 2674 (1982) and Beier et al. in *Nature* 300: 724 (1982)), enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPA 73,657.

Yeast secreted proteins generally are initially expressed as precursors bearing an N-terminal signal or leader peptide. Signal peptides generally contain a positively charged N-terminus followed by a hydrophobic core, followed by a recognition site for an enzyme known as signal peptidase. This enzyme cleaves the signal peptide from the protein during translocation. The protein is transported from the endoplasmic reticulum to the Golgi apparatus, and then follows one of a number of routes in the secretory pathway, depending on the nature of the protein. The protein may be secreted into the culture medium or may be retained on the cell surface, for example. Certain receptors that comprise extracellular, transmembrane, and cytoplasmic domains are examples of proteins that may be retained on the cell membrane, with only the extracellular domain located outside the cell.

The leader sequences of certain secreted proteins comprise peptides that are located C-terminal to the signal peptide and are processed from the mature protein of interest subsequent to cleavage of the signal peptide. Such leaders often are referred to as prepro peptides, wherein the pre region is the signal sequence and the pro region designates the remainder of the leader. One example is the yeast α-factor leader, that contains a signal peptide (including a C-terminal signal peptidase recognition site AlaLeuAla) followed by a pro region containing a basic amino acid pair LysArg that constitutes a KEX2 protease processing site, immediately followed by a peptide GluAlaGluAla at the C-terminus of the pro region. Processing of this leader involves removal of the signal peptide by signal peptidase, followed by cleavage between the Lys and Arg residues by KEX2 protease. The GluAlaGluAla residues are subsequently removed by a peptidase that is the product of the STE13 gene (Julius et al., *Cell* 32: 839 (1983)). The yeast α-factor leader is described in U.S. Pat. No. 4,546,082.

The yeast expression vector advantageously comprises DNA encoding a suitable leader or signal peptide fused to the 5' end of the DNA encoding the fusion protein. The leader peptide thus is fused to the N-terminus of the fusion protein when initially expressed, and promotes secretion of the expressed fusion protein from the cell. The leader peptide is cleaved by specific intracellular protease(s) during secretion, so that the fusion protein recovered from the culture medium does not have the leader peptide attached thereto.

Any signal or leader peptide recognized by *S. cerevisiae* cells may be employed. Examples are the leader or signal peptide of such proteins as the *S. cerevisiae* α-factor MFα1 (described in U.S. Pat. No. 4,546,082), *S. cerevisiae* invertase, encoded by the SUC2 gene (Smith et al., *Science* 229: 1219, 1985; Chang et al., *Mol. Cell. Biol.* 6: 1812, 1986), *S. cerevisiae* acid phosphatase, encoded by PH05 (Smith et al., 1985, supra; Hinnen et al. in Korhola and Valsanen, Eds., *Gene Expression in Yeast, Foundation for Biotechnological and Industrial Fermentation Research*, Vol 1, Kauppakirjapaino Oy, Helsinki, 1983, pp. 157–163), *S. carlsbergensis* α-galactosidase (the MEL1 gene product) (Hofmann and Schultz, *Gene* 101: 105, 1991), *K. lactis* killer toxin (ORF2) (Stark and Boyd, *EMBO J.* 5: 1995, 1986; Baldari et al., *EMBO J.* 6: 229, 1987), *S. cerevisiae* killer toxin (Tokunaga et al., *Nuc. Acids res.* 16: 7499, 1988), and the *S. cerevisiae* BGL2 gene product (Ackerman et al., *Gene* 110: 25, 1992). The pre or prepro region of a given leader (discussed above) may be employed.

Preferably, a signal peptide derived from a type I interleukin-1 receptor (IL-1R) signal sequence lacking its native signal peptidase recognition site is used. This signal peptide has the formula sig[Z]$_n$AlaXaIa, wherein sig represents a truncated type I interleukin-1 receptor signal sequence lacking the amino acids at positions y through -1 of the native signal sequence, wherein y is −3 or −4. The sig moiety is derived from the signal sequence of a type I interleukin-1 receptor. Such signal sequences include the human and murine type I IL-1 receptor signal sequences described in U.S. Pat. No. 5,081,228 (hereby incorporated by reference) or homologous signal peptides derived from other mammalian species.

Z represents an optional spacer peptide comprising from 1–5 amino acids, preferably 1–3 amino acids; and n is 0 or 1. Z contains neither the native signal peptidase recognition site of the interleukin-1 receptor signal sequence, nor a tripepride of the formula AlaXAla. One example of Z is a peptide encoded by a linker useful for constructing a recombinant vector, e.g., a linker containing a desired restriction site. The AlaXAla tripeptide replaces the native signal peptidase recognition site. X is an amino acid selected from the group consisting of Leu, Phe, and Gln, preferably Leu. For expression of a desired fusion protein, DNA encoding the fusion protein is fused to the 3' end of the DNA segment encoding this signal peptide.

Preferred yeast vectors can be assembled using DNA sequences from pBR322 for selection and replication in *E. coli* (Amp^r gene and origin of replication) and yeast DNA sequences including a glucose-repressible ADH2 promoter and α-factor secretion leader. The yeast α-factor leader, that directs secretion of heterologous proteins, can be inserted between the promoter and the structural gene to be expressed. See, e.g., Kurjan et al., *Cell* 30: 933 (1982), and Bitter et al., *Proc. Natl. Acad. Sci. USA* 81: 5330 (1984). The leader sequence may be modified to contain, near its 3' end, one or more useful restriction sites to facilitate fusion of the leader sequence to foreign genes.

A particularly preferred eukaryotic yeast vector for expression of GM-CSF/antigen DNA is pIXY456. pIXY456 is a derivative of the prADH2 yeast expression plasmid described by V. Price et al. in *Gene*, 55: 287–293 (1987); the phage f1 origin of replication in pIXY456 does not exist in prADH2.

Suitable yeast transformation protocols are known to those of skill in the art; an exemplary technique is described by Hinnen et al. in *Proc. Natl. Acad. Sci. USA* 75: 1929 (1978), selecting for Trp$^+$ transformants in a selective medium consisting of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine and 20 μg/ml uracil.

Host strains transformed by vectors comprising the ADH2 promoter may be grown for expression in a rich medium consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μg/ml adenine and 80 μg/ml uracil. Derepression of the ADH2 promoter occurs upon exhaustion of medium glucose. Crude yeast supernatants are harvested by centrifugation are filtered and held at 4° C. prior to further purification.

Purified fusion proteins or analogs are prepared by culturing suitable host/vector systems to express the recombinant translation products of the DNAs of the present invention, that are then purified from culture media or cell extracts. For example, supernatants from systems that secrete recombinant protein into culture media can be first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a suitable purification matrix. For example, a suitable affinity matrix can comprise a GM-CSF receptor or lectin or antibody molecule bound to a suitable support.

Fermentation of yeast that express fusion proteins as a secreted protein greatly simplifies purification. Secreted recombinant protein resulting from a large-scale fermentation can be purified by methods analogous to those disclosed by Urdal et al. in *J. Chromatog.* 296: 171 (1984). This reference describes two sequential, reverse-phase HPLC steps for purification of recombinant murine GM-CSF on a preparative HPLC column.

Fusion protein synthesized in recombinant culture is characterized by the presence of unwanted and unknown proteins (contaminants) in amounts and of a character that depend upon the purification steps taken to recover the fusion protein from the culture. These components ordinarily will be of yeast origin and preferably are present in innocuous contaminant quantities, on the order of less than about 5 percent by scanning densitometry or chromatography. Further, recombinant cell culture enables the production of the fusion protein free of proteins that may be normally associated with GM-CSF or the antigen as they are found in nature in their respective species of origin, e.g., in cells, cell exudates or body fluids.

Fusion protein compositions are prepared for administration by mixing fusion protein having the desired degree of purity with physiologically acceptable carriers. Such carriers will be nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the fusion protein with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about ten residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLE 1

Construction of muGM-CSF/Malaria Antigen Yeast Expression Vectors

Yeast expression plasmid pIXY456 was digested with the restriction enzymes Asp718 and Spe1 and the large vector fragment was purified using standard molecular biology techniques. DNA encoding the murine GM-CSF gene (muGM-CSF; *PNAS* 82: 6250 (1985)) was amplified using the polymerase chain reaction (PCR). PCR primer sequences used were SEQ ID NO 3 for the 5' primer and SEQ ID NO 4 for the 3' primer. The 5' primer included an Asp718 restriction site to fuse the muGM-CSF in frame to the Asp718 site in the α-factor leader, regenerating the 3' end of the leader. The 3' primer included a portion of the $Gly_4SerGly_5Ser$ linker (amino acid sequence Gly-Gly-Gly-Gly-Ser) and a BamH1 site.

A DNA fragment encoding the gene for the Pfs25 malarial antigen (*Nature*, 333: 74–76 (1988) and *Bio/technology* 12: 494–499 (1994)) also was generated using the polymerase chain reaction in such a way as to add the 3' end of the $Gly_4SerGly_5Ser$ linker from the BamH1 site (nucleic acids 5 through 28 of SEQ ID NO 5) at the 5' end of antigen and to add a Spe1 restriction site after the termination codon for the gene. Thus the malarial antigen PCR primer sequences used were SEQ ID NO 5 for the 5' primer and SEQ ID NO 6 for the 3' primer.

The PCR product encoding muGM-CSF+linker was purified and digested with the enzymes Asp718 and BamH1. Similarly, the PCR product encoding the Pfs25 antigen was purified and digested with the enzymes BamH1 and Spe1. These two DNA fragments were ligated into the Asp718-Spe1 cut vector described above. This created a fusion DNA construct encoding muGM-CSF-linker-Pfs25 antigen. The linker has the sequence of SEQ ID NO 1.

S. cerevisiae strain XV2181 cells were transformed by conventional techniques with muGM-CSF-linker-Pfs25 antigen DNA construct. The transformed cells were cultured in 1 liter shake flasks in 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μg/ml adenine and 80 μg/ml uracil. Derepression of the ADH2 promoter occurred when glucose is exhausted from the medium. After cultivation for about 24–28 hours to permit expression and secretion of the soluble fusion protein into the supernatant, the cells were pelleted by centrifugation and the supernatant (culture medium) was filtered.

Supernatants containing the soluble fusion protein were purified. First, they were first concentrated using a commercially available protein concentration filter (an Amicon or Millipore Pellicon ultrafiltration unit). Following the concentration step, the concentrate was purified by using nickel

agarose to select for the poly-his tail. Alternatively, reverse-phase HPLC methods analogous to those disclosed by Urdal et al. in *J. Chromatog.* 296: 171 (1984) can be used.

The filtered supernatants or purified fusion proteins were analyzed for muGM-CSF biological activity and Pfs25 antigen presence. To test for muGM-CSF activity, proliferation of the muGM-CSF factor dependent cell line FCDP-2-1D was used to measure the GM-CSF-biological activity of the supernatants or purified fusion proteins. The results for the purified muGM-CSF-linker-Pfs25 antigen fusion proteins were similar to those for the muGM-CSF clone disclosed in *PNAS* 82: 6250 (1985). The filtered supernatants and purified fusion proteins also tested positive in murine bone marrow colony assays. To test for Pfs25 antigen presence within the fusion proteins, antigen-antibody reactions with Pfs25 antigen-specific antibodies were used with positive results. After as few as one innoculation, murine animals inoculated with the muGM-CSF-linker-Pfs25 antigen fusion proteins should have significantly higher antibody titers to Pfs25 than those inoculated with Pfs25 antigen alone.

EXAMPLE 2

Construction of Other muGM-CSF/Antigen Yeast Expression Vectors

The vector from Example 1 containing the muGM CSF gene fused in-frame to a $Gly_4SerGly_5Ser$ linker and the Pfs25 antigen was then used to generate other muGM-CSF fusion expression plasmids. The fusion DNA construct encoding muGM-CSF-linker-Pfs25 antigen from Example 1 was digested with BamH1 and Spe1 and the large vector fragment containing the DNA encoding muGM-CSF and a portion of the $Gly_4SerGly_5Ser$ linker was purified. The DNA sequences encoding other antigens (e.g., the MSP1 malarial antigen disclosed by Kaslow et al. in *Molecular and Biochemical Parasitology* 63: 283–289 (1994) and *Haemophilus influenzae* outer membrane lipoprotein disclosed by Deich et al in *J. Bacteriology* 170(2): 489–498 (1988)) were amplified using the polymerase chain reaction in such a way as to create the BamH1 site at their 5' terminus and the remainder of the $Gly_4SerGly_5Ser$ linker sequence. This allowed ligation of the antigen sequence in-frame to the muGM-CSF-linker sequence at the BamH1 site. The 3' PCR primer included a Spe1 site at the 3' end after the termination codon.

S. cerevisiae strain XV2181 cells were transformed by conventional techniques with either the muGM-CSF-linker-MSP1 antigen DNA construct or the muGM-CSF-linker *H. influenzae* OMP DNA construct. The transformed cells were cultured as described above in Example 1. Supernatants containing the soluble muGM-CSF-linker-MSP 1 fusion proteins were purified as described in Example 1. Supernatant containing the soluble muGM-CSF-linker-*H. influenzae* OMP fusion proteins were purified using reverse-phase HPLC methods analogous to those disclosed by Urdal et al. in *J. Chromatog.* 296: 171 (1984).

The filtered supernatants or purified fusion proteins were analyzed for muGM-CSF biological activity as described in Example 1 with positive results. To test for *H. influenzae* OMP-antigen presence within the fusion proteins, antigen-antibody reactions using *H. influenzae* OMP-antigen-specific antibodies were used with positive results. To test for MSP1-antigen presence within the fusion proteins, antigen antibody reactions with MSP1 -antigen-specific antibodies were used with positive results.

Additionally, after one inoculation, mice inoculated with the GM-CSF-linker-MSP1 fusion protein demonstrated a significant increase in antibody titer to MSP1 over those inoculated with MSP1 alone. The increased antibody titer demonstrated the enhanced antigenic response elicited by GM-fusions. After as few as one innoculation, murine animals inoculated with the muGM CSF-linker *H. influenzae* OMP antigen fusion proteins should have significantly higher antibody titers to *H. influenzae* OMP than those inoculated with *H. influenzae* OMP antigen alone.

EXAMPLE 3

Construction of a huGM-CSF/*H. influenzae* OMP Antigen Yeast Expression Vectors

Using methods similar to those described in Example 1, vectors also are created using DNA encoding human GM-CSF (huGM-CSF; e.g., ATCC 53157) in place of muGM-CSF to create a huGM-CSF-linker-Pfs25 antigen fusion DNA construct. PCR primer sequences used are SEQ ID NO 7 for the 5' primer and SEQ ID NO 8 for the 3' primer. The 5' primer included an Asp718 restriction site to fuse the muGM-CSF in-frame to the Asp718 site in the α-factor leader, regenerating the 3' end of the leader. The 3' primer included a portion of the $Gly_4SerGly_5Ser$ linker to the BamH1 site (amino acid sequence Gly-Gly-Gly-Gly-Ser).

A DNA sequence encoding *Haemophilus influenzae* outer membrane lipoprotein disclosed by Deich et al in *J. Bacteriology* 170(2): 489–498 (1988) was generated using the polymerase chain reaction in such a way as to create the BamH1 site at the 5' terminus and the remainder of the $Gly_4SerGly_5Ser$ linker sequence. The PCR product encoding muGM-CSF-linker was purified and digested with the enzymes Asp718 and BamH1. Similarly, the PCR product encoding the *H. influenzae* OMP antigen was purified and digested with the enzymes BamH1 and Spe1. These two DNA fragments were ligated into the Asp718-Spe1 cut vector described above. This created a fusion DNA construct encoding huGM-CSF-linker-*H. influenzae* OMP antigen.

Conventional techniques and the huGM-CSF-linker-*H influenzae* OMP DNA construct were then used to transform *S. cerevisiae* cells. The transformed cells were cultured as described above in Example 1. Supernatant containing the soluble huGM-CSF-linker-*H. influenzae* OMP fusion proteins were purified using reverse-phase HPLC methods analogous to those disclosed by Urdal et al. in *J. Chromatog.* 296: 171 (1984).

The purified fusion proteins are analyzed for huGM-CSF biological activity and *H. influenzae* OMP antigen presence. To test for huGM-CSF biological activity, proliferation of the huGM-CSF factor dependent cell line TF-1 is used to measure the GM-CSF-biological activity of the secreted fusion proteins. The results for the purified huGM CSF-linker-*H. influenzae* OMP antigen fusion proteins were similar to those for huGM-CSF (ATCC 53157). The filtered supernatants and purified fusion proteins also tested positive in human bone marrow colony assays. To test for *H. influenzae* OMP antigen presence within the fusion proteins, antigen-antibody reactions using *H. influenzae* OMP-antigen-specific antibodies were used with positive results. After as few as one innoculation, animals or humans inoculated with the huGM-CSF-linker-*H. influenzae* OMP antigen fusion protein should have significantly higher antibody titers to *H. influenzae* OMP than those inoculated with *H. influenzae* OMP antigen alone.

EXAMPLE 4

Construction of huGM-CSF/Malaria Antigen Yeast Expression Vectors

Similar to the process described in Example 2, the huGM-CSF-linker-*H. influenzae* OMP antigen fusion DNA construct of Example 3 can be used to generate other huGM-CSF fusion expression plasmids. For example, the fusion DNA construct encoding huGM-CSF linker-*H. influenzae* OMP antigen from Example 3 is digested with BamH1 and Spe1 and the large vector fragment containing the DNA encoding muGM-CSF and a portion of the $Gly_4SerGly_5Ser$ linker was purified.

A DNA fragment encoding the gene for the Pfs25 malarial antigen can be generated using the polymerase chain reaction in such a way as to create the BamH1 site at the 5' terminus and the remainder of the $Gly_4SerGly_5Ser$ linker sequence. This allows ligation of the antigen sequence in-frame to the huGM-CSF-linker sequence at the BamH1 Site.

S. cerevisiae strain XV2181 cells or YIMX9 cells are transformed by conventional techniques with the huGM-CSF-linker-Pfs25 antigen. The transformed cells are cultured as described above in Example 1. Supernatants containing the soluble huGM-CSF-linker-Pfs25 antigen fusion proteins are purified using the same methods used for purification of the muGM-CSF-linker-Pfs25 antigen fusion proteins in Example 1.

The purified fusion proteins are analyzed for huGM CSF biological activity as described in Example 3. To test for Pfs25 antigen presence within the secreted fusion proteins, antigen-antibody reactions described in Example 1 are used. After as few as one innoculation, animals or humans inoculated with the huGM-CSF-linker-Pfs25 antigen fusion proteins should have significantly higher antibody titers to Pfs25 than those inoculated with Pfs25 antigen alone.

EXAMPLE 5

Construction of Alternative Linkered muGM-CSF/Malaria Antigen Yeast Expression Vectors

A yeast expression vector similar to the ones described in Example 1 above can be made with a $(Ala\ Gly\ Ser)_4$ linker instead of the $Gly_4SerGly_5Ser$ linker. As in Example 1, yeast expression plasmid pIXY456 is digested with the restriction enzymes Asp718 and Spe1 and the large vector fragment is purified using standard molecular biology techniques. DNA encoding the murine GM-CSF gene (muGM-CSF; *PNAS* 82, 6250 (1985)) is amplified using the polymerase chain reaction (PCR). Instead of SEQ ID NOs 3 and 4 in Example 1, PCR primer SEQ ID NO 3 for the 5' primer and SEQ ID NO 9 for the 3' primer are used. The 5' primer includes an Asp718 restriction site to fuse the muGM-CSF in-frame to the Asp718 site in the α-factor leader, regenerating the 5' end of the leader.

A DNA fragment encoding the gene for the Pfs25 malarial antigen also is generated using the polymerase chain reaction in such a way as to add the 3' end of the $(Ala\ Gly\ Ser)_4$ linker from the BamH1 site (nucleic acids 5 through 10 of SEQ ID NO 10) at the 5' end of antigen and to add a Spe1 restriction site after the termination codon for the gene. Thus the malarial antigen PCR primer sequences used are SEQ ID NO 10 for the 5' primer and SEQ ID NO 6 for the 3' primer.

The PCR product encoding muGM-CSF+linker is purified and digested with the enzymes Asp718 and BamH1. Similarly, the PCR product encoding the Pfs25 antigen is purified and digested with the enzymes BamH1 and Spe1. These two DNA fragments are ligated into the Asp718-Spe1 cut vector described above. This creates a fusion DNA construct encoding muGM-CSF-linker-Pfs25 antigen. The linker has the sequence of SEQ ID NO 2.

S. cerevisiae strain XV2181 cells or YIMX9 cells are transformed by conventional techniques with muGM-CSF-linker-Pfs25 antigen DNA construct. The transformed cells are cultured as described above in Example 1. Supernatants containing the soluble fusion protein are purified as described in Example 1. The purified fusion proteins are analyzed for muGM-CSF biological activity as described in Example 1. To test for Pfs25 antigen presence within the fusion proteins, antigen-antibody reactions with Pfs25 antigen-specific antibodies are used. After as few as one innoculation, murine animals inoculated with the muGM-CSF-linker Pfs25 antigen fusion proteins should have significantly higher antibody titers to Pfs25 than those inoculated with Pfs25 antigen alone.

Similar to the process described in Example 2, the muGM-CSF-linker-Pfs25 antigen fusion DNA construct of this example can be used to generate other muGM-CSF fusion expression plasmids.

EXAMPLE 6

Construction of Alternative Linkered huGM-CSF/Malaria Antigen Yeast Expression Vectors

Yeast expression vectors similar to the ones described in Example 3 above can be made with an $(Ala\ Gly\ Ser)_4$ linker instead of the $Gly_4SerGly_5Ser$ linker. As in Example 3, yeast expression plasmid pIXY456 is digested with the restriction enzymes Asp718 and Spe1 and the large vector fragment is purified using standard molecular biology techniques. DNA encoding the huGM-CSF gene is amplified using the polymerase chain reaction (PCR). Instead of SEQ ID NOs 7 and 8 in Example 3, PCR primer SEQ ID NO 7 for the 5' primer and SEQ ID NO 11 for the 3' primer are used. The 5' primer includes an Asp718 restriction site to fuse the huGM-CSF in-frame to the Asp718 site in the α-factor leader, regenerating the 5' end of the leader. The 3' primer includes a portion of the $(Ala\ Gly\ Ser)_4$ linker and a BamH1 site.

A DNA fragment encoding the gene for the Pfs25 malarial antigen also is generated using the polymerase chain reaction in such a way as to add the 3' end of the $(Ala\ Gly\ Ser)_4$ linker from the BamH1 site (nucleic acids 5 through 10 of SEQ ID NO 10) at the 5' end of antigen and to add a Spe1 restriction site after the termination codon for the gene. Thus the malarial antigen PCR primer sequences used are SEQ ID NO 10 for the 5' primer and SEQ ID NO 6 for the 3' primer.

The PCR product encoding huGM-CSF+linker is purified and digested with the enzymes Asp718 and BamH1. Similarly, the PCR product encoding the Pfs25 antigen is purified and digested with the enzymes BamH1 and Spe1. These two DNA fragments are ligated into the Asp718-Spe1 cut vector described above. This creates a fusion DNA construct encoding huGM-CSF-linker-Pfs25 antigen. The linker has the sequence of SEQ ID NO 2.

S. cerevisiae strain XV2181 cells or YIMX9 cells are transformed by conventional techniques with huGM-CSF-linker-Pfs25 antigen DNA construct. The transformed cells

are cultured as described above in Example 1. Supernatants containing the soluble fusion protein are purified as described in Example 1.

To test purified fusion proteins for huGM-CSF biological activity, proliferation of the huGM-CSF factor dependent cell line TF-1 is used to measure the huGM-CSF biological activity of the supernatants and fusion proteins. Alternatively, huGM-CSF biological activity can be used using human bone marrow colony assays. To test for the presence of Pfs25 antigen within the fusion proteins, antigen-antibody reactions described in Example 1 are used. After as few as one inoculation, animals or humans inoculated with the muGM-CSF-linker-Pfs25 antigen fusion proteins should have significantly higher antibody titers to Pfs25 than those inoculated with Pfs25 antigen alone.

Similar to the process described in Examples 2 and 4, the huGM-CSF-linker-Pfs25 antigen fusion DNA construct of this example can be used to generate other huGM-CSF fusion expression plasmids.

EXAMPLE 7

Construction of Linkerless GM-CSF-Antigen Yeast Expression Vector

Yeast expression vectors similar to the ones described in Example 1 through 6 above also can be made without a linker. As in Examples 1 through 6, yeast expression plasmid pIXY456 is digested with the restriction enzymes Asp718 and SpeI and the large vector fragment is purified using standard molecular biology techniques. DNA encoding the human or murine GM-CSF gene is amplified using the polymerase chain reaction (PCR). The 5' primer includes an Asp718 restriction site to fuse the GM-CSF in frame to the Asp718 site in the α factor leader, regenerating the 3' end of the leader.

A DNA fragment encoding the antigen of choice is generated using the polymerase chain reaction in such a way as to fuse it to GM-CSF at the 5' end of the antigen and to add a SpeI restriction site after the termination codon for the gene. Depending on sequence, a restriction site can be created near the junction of the two cDNAs. The GM-CSF and antigen DNA fragments are ligated into the Asp718-SpeI cut vector described above. This creates a fusion DNA construct encoding a linkerless human or murine GM-CSF-antigen DNA fusion construct that may be used to transform yeast host cells.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide a linkerless GM-CSF/antigen fusion by deleting the linker sequences from any one of the constructs of Examples 1 through 6. Exemplary methods of making such a deletion are disclosed by Walder et al. in *Gene* 42: 133 (1986); Bauer et al. in *Gene* 37: 73 (1985); *Craik in BioTechniques* p. 12–19 (January 1985); Smith et al. in *Genetic Engineering: Principles and Methods*, Plenum Press (1981); and U.S. Pat. Nos. 4,518,584 and 4,737,462, and are incorporated herein by reference. The construct formed may be used to transform yeast host cells.

The transformed cells are cultured as described in any of the examples provided above. After cultivation for about 24–28 hours to permit expression and secretion of the soluble fusion protein into the supernatant, the cells are pelleted by centrifugation and the supernatant (culture medium) is filtered. Supernatants containing the soluble fusion protein are purified and tested for biological activity as described in the examples above. After as few as one innoculation, murine animals or humans inoculated with the linkerless murine or human GM-antigen fusion proteins should have significantly higher antibody titers to the antigen than those inoculated with the antigen alone.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 11

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Gly Gly Gly Ser Gly Gly Gly Gly Gly Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ala Gly Ser Ala Gly Ser Ala Gly Ser Ala Gly Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATATGGTACC TTTCCATAAA AGAGACCCTG AAGCCTCTTT GGATAAAAGA GCACCCACCC 60

GCTCACCCAT C 71

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCCCGGATCC ACCGCCACCT TTTTGCACTG GTTTTTTGCA 40

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AATTGGATCC GGGGGTGGCG GCGGCTCAGC TAAGGTCACT GTCGACACCG TC 52

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(i i) MOLECULE TYPE: cDNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AATTACTAGT TCAGTGGTGG TGGTGGTGGT GTGGATCGGT AC 42

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATATGGTACC TTTGGATAAA AGAGAGGCTG AAGCCTCTTT GGATAAAAGA GCTCCAGCTA 60

GATCTCCATG T 71

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCCCGGATCC ACCGCCACCC TCCTGGACTG GCTCCCAGCA 40

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AATTGGATCC AGCAGAGCCG GCAGAGCCAG CAGAACCAGC TTTTTGGACT GGTTTTTTGC 60

A 61

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(i i) MOLECULE TYPE: cDNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AATTCCATCC GCTAAGGTCA CTGTCGACAC CGTC

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AATTCGATCC AGCAGACCCC GCAGAGCCAG CAGAACCAGC CTCCTGGACT GGCTCCCAGC 60

What is claimed is:

1. A DNA encoding a fusion protein that has the biological activity of both GM-CSF and an antigen selected from the group consisting of a tumor antigen, a microbial protein, a viral protein, and a parasite protein, comprising a DNA encoding mature GM-CSF fused to a DNA encoding the antigen, wherein the 3'-end of said GM-CSF DNA is fused to the 5'-end of said antigen DNA.

2. The DNA of claim 1, wherein said GM CSF DNA is fused to said antigen DNA via a DNA encoding a linker peptide.

3. The DNA of claim 2, wherein said linker peptide DNA encodes a peptide selected from the group consisting of (Ala Gly Ser)$_4$ and Gly$_4$SerGly$_5$Ser.

4. A recombinant expression vector for expression of a fusion protein in a yeast cell, comprising a DNA encoding a fusion protein that has the biological activity of both GM-CSF and an antigen selected from the group consisting of a tumor antigen, a microbial protein, a viral protein, and a parasite protein, comprising a DNA encoding mature GM-CSF fused to a DNA encoding the antigen, wherein the 3' end of said GM-CSF DNA is fused to the 5'-end of said antigen DNA, operably linked to a promoter and a secretion signal.

5. The recombinant expression vector of claim 4, wherein said promoter is ADH2 and said secretion signal is selected from the group consisting of a yeast α factor leader and a type I interleukin-1 receptor (IL-1R) signal sequence lacking its native signal peptidase recognition site.

6. A yeast host cell transformed or transfected with an expression vector according to claim 4.

7. The host cell of claim 6, wherein said host cell is *Saccharomyces cerevisiae*.

8. A process for preparing a fusion protein comprising GM-CSF and an antigen, comprising culturing a yeast host cell according to claim 6 under conditions promoting expression and recovering a polypeptide from the culture that has the biological activity of both GM CSF and said antigen.

9. A recombinant expression vector for expression of a fusion protein in a yeast cell, comprising a DNA encoding a fusion protein that has the biological activity of both GM-CSF and an antigen selected from the group consisting of a tumor antigen, a microbial protein, a viral protein, and a parasite protein, comprising a DNA encoding mature GM-CSF fused to a DNA encoding the antigen, wherein the 3'-end of said GM-CSF DNA is fused to the 5'-end of said antigen DNA, operably linked to a promoter and a secretion signal, wherein said GM-CSF DNA is fused to said antigen DNA via a DNA encoding a linker peptide.

10. The recombinant expression vector of claim 9, wherein said promoter is ADH2 and said secretion signal is selected from the group consisting of a yeast α-factor leader and a type I interleukin-1 receptor (IL-1R) signal sequence lacking its native signal peptidase recognition site.

11. A yeast host cell transformed or transfected with an expression vector according to claim 6.

12. The host cell of claim 11, wherein said host cell is *Saccharomyces cerevisiae*.

13. A process for preparing a fusion protein comprising GM-CSF and an antigen, comprising culturing a yeast host cell according to claim 11 under conditions promoting expression and recovering a polypeptide from the culture that has the biological activity of both GM-CSF and said antigen.

14. A method of making a GM-CSF/antigen fusion protein that has the biological activity of both GM-CSF and said antigen, wherein said antigen is selected from the group consisting of a tumor antigen, a microbial proteins a viral protein, and a parasite protein, comprising the steps of:

ligating the 3'-end of a DNA encoding mature GM-CSF to the 5'-end of a DNA encoding an antigen;

linking said ligated DNA to regulatory elements that are responsible for expression of DNA into a single biologically active protein;

inserting said ligated DNA into a yeast host cell;

culturing said yeast host cell under conditions promoting expression; and

recovering said fusion protein from said culture.

15. The method of claim 14, wherein GM-CSF DNA is ligated to said antigen DNA via a DNA encoding a linker peptide.

16. The method of claim 15, wherein said linker peptide is selected from the group consisting of $(Ala\ Gly\ Ser)_6$ and $Gly_4SerGly_3Ser$.

17. The method of claim 14, wherein said regulatory elements are selected from the group consisting of a transcriptional promoter, an optional sequence to control transcription, and a stop codon.

18. The method of claim 17, wherein said promoter is ADH2.

19. The method of claim 17, wherein said regulatory elements further comprise a DNA encoding a secretion signal selected from the group consisting of a yeast α-factor leader and a type I interleukin-1 receptor (IL-1R) signal sequence lacking its native signal peptidase recognition site.

20. The method of claim 14, wherein said yeast host cell is *Saccharomyces cerevisiae*

21. A method of making a GM-CSF/antigen fusion protein that has the biological activity of both GM-CSF and said

antigen, wherein said antigen is selected from the group consisting of a tumor antigen, a microbial protein, a viral protein, and a parasite protein, comprising the steps of:

culturing said yeast cell transformed with an expression vector comprising a promoter, a DNA encoding mature GM-CSF fused to the 5'-end of a DNA encoding an antigen, and a stop codon under conditions that promote expression of said fusion protein; and

recovering said fusion protein from said culture.

22. The method of claim 21, wherein said promoter is ADH2.

23. The method of claim 21, wherein said expression vector further comprises a DNA encoding a secretion signal selected from the group consisting of a yeast α-factor leader and a type I interleukin 1 receptor (IL-1R) signal sequence lacking its native signal peptidase recognition site.

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